FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
UCIVN-014US	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
To Be Determined	10/018534

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/16895	19 June 2000	17 June 1999 & 14 July 1999

TITLE OF INVENTION

CONTINUOUS CARDIAC PERFUSION PRESERVATION WITH PEG-HB FOR IMPROVED HYPOTHERMIC STORAGE

APPLICANT(S) FOR DO/EO/US

REGENTS OF THE UNIVERSITY OF CALIFORNIA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☒ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☐ Other items or information:

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/018534 To Be Determined	PCT/US00/16895	UCIVN-014US

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00	
☒	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	$100.00
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	30 - 20 =	10	x $18.00	$180.00
Independent claims	- 3 =	0	x $84.00	$0.00
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00

TOTAL OF ABOVE CALCULATIONS =	$280.00
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	$0.00
SUBTOTAL =	$280.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	$0.00
TOTAL NATIONAL FEE =	$280.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	$0.00
TOTAL FEES ENCLOSED =	$280.00
Amount to be: refunded	$
charged	$

a. ☒ A check in the amount of _____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0878** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Robert D. Buyan
Stout, Uxa, Buyan & Mullins, LLP
4 Venture, Suite 300
Irvine CA 92618

SIGNATURE

Robert D. Buyan
NAME

32,460
REGISTRATION NUMBER

December 17, 2001
DATE

CONTINUOUS CARDIAC PERFUSION PRESERVATION WITH PEG-HB FOR IMPROVED HYPOTHERMIC STORAGE

Be it known that we, Danny L. Serna Jr., Jeffrey C. Milliken, and Ralph E. Purdy, each citizens of the United States, have invented a new and useful method and apparatus for continuous cardiac perfusion preservation with PEG-Hb for improved hypothermic storage of which the following is a specification.

Donor organ preservation for transplantation is performed using ischemic hypothermic immersion storage in saline solution. Preservation time for the donor cardiac allograft, for example, is limited to a maximum of 4 to 6 hours using this technique. Hypothermic perfusion preservation with an oxygen carrying hemoglobin solution should extend preservation times and decrease ischemic injury of transplantable organs. Perfusion preservation using the invention will also allow sufficient time for complex tissue typing, allow better donor-recipient matching, and allow for transportation of organs to more distant sites.

Prior art bearing on the invention includes PEG-Hb which Enzon, Inc as described in U.S. Patent 5,312,808. The invention differs from prior art in terms of the composition of the solution. The invention contains PEG-Hb as one of numerous components. In addition to PEG-Hb, the invention contains human albumin, dextrose, heparin sodium, lidocaine HCl, $MgSO_4$, KCl, $CaCl_2$, THAM, NaCl, NaHCO3, Na_3P0_4, without which PEG-Hb is lethal to the myocardium and cannot be used for the purpose of effective organ preservation.

In the past, the problem of cardiac allograft preservation was accomplished by hypothermic immersion storage of the allograft in cardioplegia or saline solution. The disadvantage of this technique was the lack of delivery of oxygen, nutrition, and electrolytes to the donor organ allograft. Use of PEG-Hb alone is lethal to the myocardium and cannot be used for the purpose of effective organ preservation. An electrolyte and nutritional formulation was developed that included PEG-Hb and was found to improve and extend myocardial preservation times above that achieved by standard techniques.

The invention includes a composition of matter, namely a polyethylene glycol coated bovine hemoglobin based solution for the purpose of ex vivo donor

organ preservation and the use of the same. The purpose of the solution is to preserve donor human and animal organs, *ex vivo*, prior to transplantation. The fundamental principle of the solution is to provide an oxygen, nutritional and electrolyte environment to the tissue of the donor organ that is conducive to *ex vivo* preservation such that the donor organ will regain acceptable function post transplantation.

The invention is a product, composition, and method of use and is set forth in greater specificity in Appendices 1 – 3 which are incorporated herein by reference as if set out in its entirety with Appendix 3 being the most current implementation of the invention.

The advantages of the invention include provision of oxygen, a carbohydrate energy source, continuous metabolite washout, and continuous perfusion with an isotonic, normokalemic, hypocalcemic solution that drastically improves myocardial preservation over current techniques considered the standard of care. Hypothermic perfusion preservation of the rabbit heart using the invention for periods of 8 hours has been shown to improve myocardial preservation and left ventricular function compared to 4 hours of hypothermic immersion storage in saline solution, which is considered to be the standard of care. Hypothermic perfusion preservation of the rabbit heart using the invention for periods of 8 hours has also been shown to produce left ventricular function that trends toward superiority over fresh control rabbit hearts immediately after removal from the chest.

The illustrated composition of the PEG-Hb based preservation fluids is as follows: 3% PEG-Hb, KCL (4.7 mEq/L), NaCl (148.7 mmol/L), NaPO4 (2.5 mmol/L), NaHCO3 (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), Tromethamine (THAM) solution (7.3 cc/L).

The composition of the crystalloid preservation solution is as follows: KCL (4.7 mEq/L), NaCl (150.7 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L),

human albumin (1.5 gm/L), human insulin (30.6 units/L), and Tromethamine (THAM) solution (7.3 cc/L).

During *ex vivo* cardiac preservation, the pH of the invention is maintained at 7.1, as measured at 37°C, and 7.4 as measured at 20°C. The pO2 is maintained above 600 mm Hg. New Zealand White rabbits were used to obtain data to support this disclosure.

The proposed use of the invention is for the *ex vivo* preservation of human and animal donor organ allografts during transportation from the donor to the recipient for the purpose of transplantation. In addition to its use for ex vivo myocardial preservation, this PEG-Hb solution has tremendous potential utility for in vivo myocardial preservation during open heart surgery as well as a blood substitute or blood replacement during or following surgery of any sort, including open heart surgery.

By increasing the potassium concentration of the solution to reflect intracellular levels, this solution could very well be useful for the purposes of cardioplegia or hypothermic cardiac arrest as well as myocardial preservation during open-heart surgery. The solution could be administered in order to effect and maintain myocardial arrest as well as improve myocardial preservation during open heart surgery.

The current formulation of the PEG-Hb solution would likely be extremely effective for the purposes of intravascular volume replacement, blood substitution, and as an alternative to blood transfusion during or after surgery of any sort including, but not limited to open heart surgery, and including trauma induced blood loss.

Many alterations and modifications may be made by those having ordinary skill in the art without departing from the spirit and scope of the invention. Therefore, it must be understood that the illustrated embodiment has been set forth only for the purposes of example and that it should not be taken as limiting the invention which could be more broadly or narrowly defined later by patent claims.

The words used in this specification to describe the invention and its various embodiments are to be understood not only in the sense of their

commonly defined meanings, but to include by special definition in this specification structure, material or acts beyond the scope of the commonly defined meanings. Thus if an element can be understood in the context of this specification as including more than one meaning, then its use in later in a claim must be understood as being generic to all possible meanings supported by the specification and by the word itself.

The definitions of the words or elements of the following claims are, therefore, defined in this specification to include not only the combination of elements which are literally set forth, but all equivalent structure, material or acts for performing substantially the same function in substantially the same way to obtain substantially the same result. In this sense it is therefore contemplated that an equivalent substitution of two or more elements may be made for any one of the elements in later defined claims or that a single element may be substituted for two or more elements in later defined claims.

Insubstantial changes from the claimed subject matter as viewed by a person with ordinary skill in the art, now known or later devised, are expressly contemplated as being equivalently within the scope of the invention. Therefore, obvious substitutions now or later known to one with ordinary skill in the art are defined to be within the scope of the defined elements.

The invention is thus to be understood to include what is specifically illustrated and described above, what is conceptionally equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention.

APPENDIX 1

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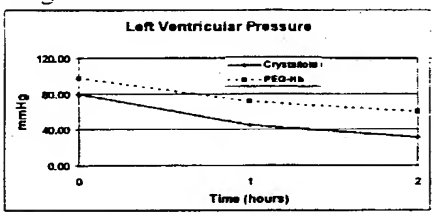
CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING PEG-HEMOGLOBIN VS. CRYSTALLOID PERFUSION

Efforts to extend myocardial preservation for transplantation by crystalloid perfusion has been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with an oxygen carrying hemoglobin solution may extend preservation times. The purpose of this study was to compare cardiac function after continuous perfusion with PEG-Hemoglobin (Hb) vs. a physiologic crystalloid perfusate. The hearts of 9 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=4) hearts were continuously perfused with PEG-Hb (Enzon Inc., N.J.) at 20°C and 30 mmHg for 8 hours. Group II (n=5) hearts were continuously perfused with crystalloid perfusion for 8 hours at 20°C. Cardiac function was measured with a left ventricular balloon at 0, 1, and 2 hours after transfer to a standard crystalloid Langendorff circuit.



Heart rate was the same for group I and II through the testing period (89.6 vs. 91.1, p=0.57). Developed LV pressure (systolic minus diastolic) at 0.6cc LV volume was greater in Group I (76.17±19.2 mmHg), than in Group II (52.0±25.21, p=0.021). Maximum +dP/dT at 0.6 cc LV volume was greater in Group I (854.47±381.8mmHg/sec) than in Group II (485.10±284.14 mmHg/sec, p=0.025). Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II. Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20°C yields left ventricular function is superior to 8-hr crystalloid perfusion, despite similar myocardial edema. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation.

DL Serna, LL Powell, WC Wallace, J West, C Kahwaji, G Cogert, P Smulowitz, E Steward, R Purdy, JC Milliken. UC Irvine.

24 Hour Cardiac Perfusion Preservation Using a Novel PEG-Hemoglobin Solution

Dan L. Serna, Ledford L. Powell, Peter Smulowitz, Gerry Beckham, Chadi I. Kahwaji, Blanding U Jones, Peter Connolly, Vini Shrivastata, Yannelly Perez, Adam Farber, Justin West, Earl Steward, Ralph Purdy, Jeffrey C. Milliken.

Cardiac preservation for transplantation is limited by ischemic hypothermic storage of 4 to 6 hours. Hypothermic perfusion preservation using a novel oxygen carrying hemoglobin solution may extend preservation times and decrease ischemic injury. The purpose of this study was to compare cardiac function after 24 hrs of continuous hypothermic perfusion with a PEG-Hemoglobin (Hb) solution to the clinical standard of hypothermic ischemic preservation.
Methods: The hearts of 25 anesthetized and intubuted NZW rabbits were harvested after cold cardioplegic arrest. Group I(n=7) hearts were perfused with a PEG-Hb solution at 20C and 30 mmHg for 24 hours. Group II(n=10) hearts were preserved by cold ischemic storage for 4 hours at 4C, and Group III(n=8) were tested immediately after harvest. LV function was measured in the non-working state immediately and 2 hours after transfer to a standard crystalloid Langendorff circuit.
Results: Developed LV pressure at 0.5cc LV volume was similar in Group I (54.2 ±2.6mmHg) and Group II (49.1±5.4mmHg,p=.5) but greater in Group III (69.4±5.1mmHg, p=.02). Maximum-dp/dT at 0.5 cc LV volume was similar in Group I(-398.1±19.0 mmHg/sec), Group II (-354.8±49.1mmHg/sec,p=.2) and Group III(-456.2±44.1mmHg/sec,p=.7). Maximum +dp/dT at 0.5cc LV volume was also similar in Group I (660.3±49.5mmHg/sec), Group II (428.4±54.9mmHg/sec,p=.3) and Group III(514.6±48.9mmHg/sec,p=.6).
Conclusions: Continuous perfusion preservation of rabbit hearts for 24 hrs with this novel PEG-Hb solution at 30 mmHg and 20 C yields left ventricular function that is similar to 4 hrs of ischemic hypothermic storage and to that of fresh control hearts. Extended cardiac perfusion preservation with this PEG-Hb solution deserves further investigation in large animal transplant models.

APPENDIX 2

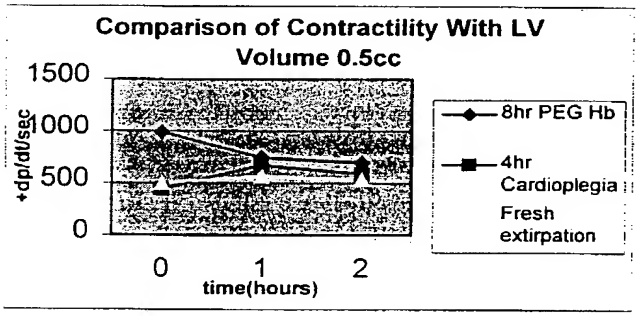
PERFUSION PRESERVATION WITH NOVEL PEG-HB SOLUTION MAY ALLOW RECOVERY OF FUNCTION IN THE DONOR RABBIT CARDIAC ALLOGRAFT AFTER CARDIOPLEGIC ARREST.

Blanding U. Jones, MD, Dan L. Serna, MD, Gerry Beckham, BS, Peter Smulowitz, BS, Adam Farber, BS, Chad Kahwaji, BS, Earl Steward, BS, Ralph E. Purdy, Ph. D., and Jeffrey C. Milliken, MD

INTRODUCTION: The clinical standard of hypothermic storage preservation of the cardiac allograft imposes an ischemic insult to the allograft and limits preservation of the allograft to 4 to 6 hours. Continuous hypothermic perfusion preservation with a novel, oxygen carrying, PEG-hemoglobin (Hb) solution may decrease ischemic injury and allow better and lengthier preservation of cardiac function than the clinical standard. The purpose of this study was to compare cardiac function after 8 hrs of continuous hypothermic perfusion using a novel PEG-Hb solution to 4 hrs of hypothermic ischemic storage preservation.

METHODS: The hearts of 28 anesthetized and intubated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused via the aortic root with a novel PEG-Hb solution at 20°C and 30 mmHg for 8 hours. PO_2 was maintained greater than 500 mmHg during the preservation phase. Group II (n=10) hearts were preserved by cold ischemic storage for 4 hours at 4°C. Group III hearts (n=8) were tested immediately after their harvest. Left ventricular function was measured at 37°C in the non-working state 15 min after transfer to a standard crystalloid Langendorff circuit.

RESULTS: Developed LV pressure at 0.5cc LV volume was greater in Group I (75.7±10.3mmHg) than Group II (49.1±5.4mmHg,p=.04) and similar to Group III (69.4±5.1mmHg, p=.6). Maximum-dp/dT at 0.5 cc LV volume was greater in Group I(-610.6±68.4mmHg/sec) than Group II (-354.8±49.1mmHg/sec,p=.01) and trended toward superiority over Group III(-456.2±44.1mmHg/sec,p=.09). Maximum +dp/dT at 0.5cc LV volume was greater in Group I (964.9±156.6mmHg/sec) than both Group II (428.4±54.9mmHg/sec,p=.004) and Group II (514.6±48.9mmHg/sec, p=.02).



CONCLUSIONS: Continuous perfusion preservation of the rabbit heart for 8 hrs with this PEG-Hb solution at 30 mmHg and 20°C yields left ventricular function that is superior to 4 hrs of ischemic hypothermic storage. Furthermore, return of cardiac function after perfusion preservation using this PEG-Hb solution may be superior to that obtained in freshly arrested hearts. These data suggest there may occur some recovery of myocardial function during perfusion preservation with this PEG-Hb solution after the ischemic insult of cardioplegic arrest. Perfusion preservation using this PEG-Hemoglobin solution may also be more useful than hypothermic ischemic storage in the reanimation of non-beating heart donors. Continuous perfusion preservation using this PEG-Hb solution deserves further investigation in large animal transplant models.

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APPENDIX 3

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING PEG-HEMOGLOBIN VS. CRYSTALLOID PERFUSION

Dan L. Serna, MD, Ledford L. Powell, MD, Chadi Kahwaji, BS, William C. Wallace, MD, Justin West, BA, Greg Cogert, BS, Peter Smulowitz, BS, Earl Steward, MD, Ralph E. Purdy, PhD, Jeffrey C. Milliken, MD. Division of Cardiothoracic Surgery, and Department of Pharmacology. UC Irvine Medical Center, Orange, CA.

Corresponding Author:

Jeffrey C. Milliken, MD

UC Irvine Medical Center

Division of Cardiothoracic Surgery

101 The City Drive, Bldg. 53, Rm. 117

Orange, CA 92868

(714) 456-3634 – office

(714) 456-8870 – fax

jcmillik@uci.edu- email

Running Head: Ex vivo Cardiac Preservation using PEG-Hemoglobin Solution.

Presented at the 45th Annual Conference, American Society for Artificial Internal Organs, June 2-5,1999.

ABSTRACT

Introduction: Efforts to extend myocardial preservation for transplantation by crystalloid perfusion have been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with a polyethylene glycol (PEG) conjugated hemoglobin solution may extend preservation times. The purpose of this study was to compare cardiac function after continuous perfusion using a hypocalcemic normokalemic crystalloid perfusate with and without the addition of PEG-Hemoglobin (Hb).

Methods: The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a hypocalcemic, normokalemic 3% bovine PEG-Hb solution at 20°C and 30 mmHg for 8 hours. Group II (n=10) hearts were continuously perfused with an identical crystalloid solution without PEG-Hb for 8 hours under the same conditions as Group I hearts. Cardiac function was measured with a left ventricular force transducer after transfer to a standard crystalloid Langendorff circuit at 37 °C and an aortic root pressure of 59 mmHg.

Results: After 8 hours of perfusion preservation, heart rate was similar for groups I and II (p=NS). Coronary blood flow after preservation and during preservation was similar between PEG-Hb and crystalloid preserved hearts (p=NS). Left ventricular developed pressure, peak dP/d*t*, and peak -dP/d*t* were superior in hearts preserved with PEG-Hb. Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hours with a hypocalcemic normokalemic PEG-Hb based solution at 30 mmHg and 20°C yields left ventricular function that is superior to perfusion a similar crystalloid solution without PEG-Hb, despite similar myocardial edema and coronary flow. Extended cardiac perfusion preservation with this PEG-Hb

based solution deserves further study including comparison to traditional cardioplegic preservation solutions.

INTRODUCTION

The current method of donor heart preservation for clinical transplantation involves cold cardioplegic arrest and storage at near freezing temperatures. Because of ongoing ischemia, this preservation technique prohibits extended storage of donor organs, use of advanced methods of tissue typing, and delivery of donor hearts over long distances. The current preservation technique may also lead to irreversible graft damage. Preservation by continuous coronary artery perfusion allows for greater preservation times than hypothermic ischemic preservation (1). Continuous coronary artery perfusion allows for an ongoing supply of substrate as well as removal of metabolic waste products. Three general types of solution have been examined for their efficacy as cardiac preservation agents. Perfusion with crystalloid, cardioplegia-type solutions have shown limited promise (2-6). Perfusion preservation using these solutions has been limited by edema and compromised cardiac function (2-6). Similarly, studies examining perfluorocarbon emulsions as perfusion preservation media for the donor heart have produced mixed results (1,7-10). Further, perfluorochemicals are expensive and have questionable safety profiles when used systemically (7-9).

Hemoglobin-based blood substitutes have more recently been developed for use as blood replacements in trauma and surgery. Use of these solutions as organ preservation solutions may lengthen the window of *ex vivo* cardiac preservation with a concomitant decrease in ischemia. We hypothesized that hypothermic perfusion preservation with a hypocalcemic, normokalemic, polyethylene glycol conjugated hemoglobin (PEG-Hb) based solution over 8 hours would preserve left ventricular function above that obtained with a chemically identical crystalloid

solution without PEG-Hb. We suspect that this PEG-Hb solution may optimize the donor heart during its preservation period.

The purpose of this study is to compare *ex vivo* adult rabbit heart preservation after continuous coronary artery perfusion using a hypocalcemic, normokalemic PEG-Hb solution versus an identical hypocalcemic, normokalemic crystalloid solution not containing PEG-Hb. This work will lay the foundation for future investigation comparing perfusion preservation with PEG-Hb based solutions to hypothermic ischemic storage preservation using traditional cardioplegic solutions as well as PEG-Hb solutions containing specific enhancers of myocardial preservation.

METHODS

All animals received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23). The Institutional Animal Care and Use Committee of the University of California, Irvine, approved animal procedures.

Experimental Design

The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a normokalemic hypocalcemic bovine PEG-Hb based solution at 20°C and 30 mmHg of aortic root pressure for 8 hours. Group II (n=10) hearts were identically preserved with a crystalloid solution identical in composition to Group I hearts, but without the addition of PEG-Hb.

Cardiac Procurement

Twenty adult male New Zealand White rabbits (3 to 3.5 kg) were anesthetized using an intramuscular injection of 50mg ketamine and 5mg xylazine per kilogram. Lactated Ringers

solution was infused through an IV catheter in a marginal ear vein at a rate of 5 to 15 cc/hr. The rabbits were mechanically ventilated using a Servo Animal Ventilator (model #900C, Siemens-Elema, Sweden). Anesthesia was maintained with intravenous ketamine/xylazine in a 1:1 ratio. A median sternotomy followed by a longitudinal pericardial incision was performed, exposing the heart and mediastinal vessels. All rabbits received 1,000 U heparin sodium/kg IV. The innominate artery, the aortic arch between the brachiocephalic trunk and left carotid artery, as well as the inferior and superior vena cava were the identified and isolated. Upon ligation of the inferior and superior vena cava, the innominate artery was cannulated using an 18 Ga angiocatheter. 60 cc of hypothermic cardioplegia solution (2-4°C) was administered to the coronary arteries via the innominate artery over 3 minutes. An arteriotomy was made in the pulmonary trunk to decompress the right ventricle. Hypothermic normal saline (2-4°C) was used to cool the heart during cardioplegia infusion. The heart was quickly excised and placed in cold saline (4°C) for further dissection. The heart was trimmed of excess soft tissue including lungs, trachea, and thymus. All hearts were placed onto the preservation circuit by cannulation at the ascending aorta. Coronary perfusion was begun within 5 minutes of cardiectomy. All hearts were preserved for 8 hours by continuous coronary artery perfusion. Aortic root pressure was maintained at 30 mmHg. Temperature of the perfusate was maintained at 20°C. All hearts were perfused and immersed in the respective preservation solutions for the entire 8-hour preservation period. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to the preservation circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. The preservation circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus Inc., Eden Prairie, MN), an adult membrane oxygenator

(Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA) which was circulated through the membrane oxygenator (figure 1).

Preservation Solutions

The composition of the PEG-Hb based preservation fluids is as follows: 3% bovine PEG-Hb, KCL (4.7 mEq/L), NaCl (148.7 mmol/L), NaH2PO4 (2.5 mmol/L), NaHCO3 (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), Tromethamine (THAM) solution (7.3 cc/L). The osmolality of the 3% PEG-Hb solution is 324 mOsm/kg.

The composition of the crystalloid preservation solution is as follows: KCL (4.7 mEq/L), NaCl (150.7 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6 units/L), and Tromethamine (THAM) solution (7.3 cc/L). The osmolality of the crystalloid preservation solution is 324 mOsm/kg.

Postpreservation Assessment of Cardiac Function:

At the end of the 8-hour preservation period, all hearts were transferred to an isolated heart perfusion apparatus for purposes of data collection. Coronary perfusion via the aortic root was immediately begun at 37°C and 59 mmHg aortic root pressure. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to this circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. After 15 minutes

of coronary perfusion in this position, coronary flow, heart rate, left ventricular developed pressure (LVP), peak dP/d*t*, and peak –dP/d*t* were measured. Coronary flow and heart rate were measured every 15 minutes for 2 hours. LVP, peak dP/d*t*, and peak –dP/d*t* were measured again at 75 and 135 minutes following transfer to the second circuit. Heart rate was measured by counting left ventricular contractions over the course of one minute. Coronary flow was measured by collecting the effluent that exited from the pulmonary artery over course of one minute. LVP, peak dP/d*t*, and peak –dP/d*t* were measured in the beating, nonworking position during continuous coronary artery perfusion. Developed left ventricular pressure (systolic minus diastolic) and peak rates of left ventricular pressure development (dP/dt_{max}) and relaxation ($-dP/dt_{max}$) were measured using a left ventricular force transducer (Biopac Systems, Inc., Santa Barbara, CA). Data from the LV force transducer was digitized using an analog to digital converter (Biopac Systems, Inc., Santa Barbara, CA) and analyzed using Acknowledge software (Version 3.2.6, Biopac Systems, Inc., Santa Barbara, CA) and a desktop computer (Nexstar, Fremont, CA). The testing circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus Inc., Eden Prairie, MN), an adult membrane oxygenator (Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA), which was circulated through the membrane oxygenator.

Testing Solution: Left ventricular function was assessed in all hearts in both groups using a standard physiologic crystalloid solution. The composition was as follows: KCL (4.7 mEq/L), NaCl (151.5 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (2.0 mEq/L), lidocaine HCl (12.5 mg/L),

heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human insulin (30.7 units/L), and Tromethamine (THAM) solution (6.1 cc/L).

Measurement of ventricular water content: After 135 minutes of retrograde aortic perfusion on the testing circuit, the ventricular myocardium of the initial 5 hearts in each group was dissected free of atria and other soft tissue. The left ventricular myocardium was weighed before and after desiccation at 110°C.

Data Analysis

Data are reported as means ± SE. Statistical analysis was performed using Systal 7.0.1 software package (SPSS, Inc., Chicago, IL). The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant.

Materials

Bovine PEG-Hb was obtained from Enzon, Inc. (Piscataway, NJ) in a solution containing 6% PEG-Hb, 5 mM NaH2PO4, 5 mM NaHCO3, and 150 mM NaCl. Polyethylene glycol (PEG) conjugated bovine Hb (PEG-Hb) was prepared by the isolation of hemoglobin from bovine red blood cells obtained from a closed herd. The material was purified and each Hb molecule modified with approximately 12 succinimidyl carbonate polyethylene glycol strands (5000 daltons) to yield a 6% (g/dL) Hb solution with methemoglobin less than 5% of total hemoglobin, endotoxin less than 0.5 EU/mL, and viscosity 3.1 cP at 37°C. Normal saline solution (0.9% NaCl) was obtained from Baxter Health Care (Irvine, CA). Solutions were monitored using a blood gas analyzer (288 Blood Gas System, Ciba-Corning Diagnostics Corp., Medfield, MA), an Automated Coagulation Timer (Medtronic Hemotec, Inc., Englewood, CO) and a blood glucose meter (Lifescan, Inc., Milpitas, CA). Membrane oxygenators were obtained from Sarns/3M Health Care, Inc. (Ann Arbor, MI).

RESULTS

Developed LV pressure: Developed LV pressure at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes after the end of preservation (p=0.46, figure 2). However, developed LV pressure at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 (p=0.006) and 135 minutes (p=0.002) after the end of preservation.

Maximum rate of LV contraction: Peak dP/dt_{max} at 0.5 cc LV volume trended toward superiority amongst hearts preserved using PEG-Hb solution compared to crystalloid preserved hearts, at 15 minutes after the end of preservation (p=0.10, figure 3). However, peak dP/dt_{max} at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 (p=0.01) and 135 minutes (p=0.001) after the end of preservation.

Maximum rate of LV relaxation: Peak - dP/dt_{max} at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes (p=0.27) after the end of preservation and trended toward superiority at 75 minutes after the end of preservation (p=0.07, figure 4). Peak - dP/dt_{max} at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 135 minutes (p=0.006) after the end of preservation.

Ventricular Water Content: Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Coronary Flow: Coronary flow after preservation was similar between PEG-Hb and crystalloid preserved hearts.

Heart Rate: Heart rate was the same for group I and II through the testing period (p=NS, Table 1).

CONCLUSIONS

There are two general techniques of *ex vivo* cardiac preservation for transplantation. The standard of care and commonly used technique is hypothermic ischemic immersion storage of the donor cardiac allograft. The second method of cardiac preservation is coronary perfusion preservation. These two methods can and have been used in combination with improved results. Perfusion preservation is superior to immersion preservation because it allows for the continuous washout of metabolic waste products, as well as the delivery of nutrients, metabolic substrate, and oxygen to the myocardium (1). For several reasons, perfusion preservation has not been applied clinically to *ex vivo* cardiac preservation for transplantation. First, a user friendly, practical, and portable perfusion preservation device is not currently available. Second, most research into perfusion preservation to date has been performed using crystalloid cardioplegia solutions and perfluorocarbons. Crystalloid cardioplegia solutions, unfortunately, carry very little oxygen and hence their use is associated with considerable ischemic injury to the donor organ (2-6). Perfluorocarbon based solutions have demonstrated mixed results for the purpose of cardiac preservation and are extremely expensive (1, 7-10).

Perfusion preservation using stroma-free hemoglobin based solutions represents an innovative means of *ex vivo* cardiac preservation. Stroma-free hemoglobins were initially developed as blood substitutes for use in the treatment of life threatening hemorrhage secondary to trauma. There is strong interest among transplant scientists in the potential for these solutions as organ preservation solutions. The purpose of this study was to assess the utility of perfusion preservation using normokalemic hypocalcemic polyethylene glycol coated bovine hemoglobin based solution.

The superior organ preservation results of the PEG-Hb preserved hearts in this study are probably a result of a combination of both an oncotic and oxygen delivery effect of PEG-Hb. Data supporting an oxygen delivery effect of PEG-Hb has otherwise been obtained using exchange-transfusion in a rat model (11). Rats were exchange-transfused up to an 85% hematocrit reduction with either PEG-Hb, PEG-mHb (50%-methemoglobin), PEG-carbon monoxide hemoglobin (PEG-COHb), or PEG-human serum albumin (PEG-HSA). Survival at twenty-four hours after transfusion was 79 % in the PEG-Hb group, 30 % in the PEG-mHb group, and 0% for both PEG-COHb and PEG-HSA. Despite similar plasma expansion properties of the 4 solutions, the solution with greatest oxygen delivery capability led to greatest survival.

On a per gram basis, the oxygen carrying capacity of PEG-Hb is the same as would be found with unmodified tetrameric bovine Hb. PEGylation of Hb involves the covalent attachment of polyethylene glycol to stroma-free Hb tetramers. PEGylation does not appear to change the total oxygen carrying capacity of the Hb, but PEGylation does appear to alter the nature of oxygen transport (12). For example, because of its larger particle size, PEG-Hb remains within the vascular space for longer than otherwise unmodified Hb (13). In addition, PEGylation alters the oxygen affinity of bovine hemoglobin. The P_{50} of bovine PEG-Hb is 15 torr at 37°C (14). Clearly, this is a relatively low P_{50}. Such high oxygen affinity begs the question of the ability of PEG-Hb to effectively deliver oxygen under normothermic and hypothermic conditions. Bovine PEG-Hb has been shown using the rat model to provide better tissue oxygenation than stroma-free bovine Hb (P_{50} – 26 torr) or cross-linked bovine Hb (P_{50} – 48 torr), both of which have lower affinity for oxygen than does PEG-Hb and therefore should theoretically be better tissue oxygenators (14). Furthermore, we also know that bovine Hb is unlike human Hb in that it does not require 2,3-diphosphoglycerate to lower its oxygen affinity,

but rather requires only chloride ions, which are present in the PEG-Hb preservation solution (15). Finally, the Bohr effect is more pronounced in bovine Hb than human Hb, which would theoretically allow better delivery of oxygen at lower pH and temperature (16).

The oncotic pressure of PEG-Hb is greatly enhanced by the conjugation of PEG to surface amino acid groups of the Hb. A 3 gm/dL solution, as used in this study, has a colloid osmotic pressure of approximately 39 mm Hg (17). In comparison, similar concentrations of human serum albumin and purified human hemoglobin A_0 have colloid osmotic pressures of 9 mm Hg and 9 mm Hg, respectively (17). The amount of human serum albumin used in both preservation solutions in this study, 0.15 gm/dL, has an oncotic pressure on the order of 1 mm Hg (17). The average calculated molecular weight for unmodified and intramolecularly cross-linked human tetramers is 65,300 ± 3500 compared to 117,000 for bovine PEG-Hb. When added to Bretschneider's HTK cardioplegic solution, PEG is associated with improved recovery of left ventricular function as well as less myocardial edema (18), and it is likely that the onconicity of the PEG solution plays an important role. The mechanism of action of PEG may also involve suppression of lipid peroxidation (18).

The preservation solution was made hypocalcemic because the intracellular accumulation of calcium during ischemia and reperfusion is associated with cellular injury (19-23) and a hypoxically stressed heart may be protected by a hypocalcemic solution (19). The solution was normokalemic in order to keep the heart beating, since a beating heart may be less susceptible to edema. Finally, the preservation solution was slightly hypermagnesemic because magnesium inhibits the membrane transport of calcium, and thus intracellular accumulation of calcium, which should help to prevent the deleterious effects of calcium (24-27). Magnesium has been

shown to attenuate deleterious effects of calcium in ischemic piglet hearts, which are more sensitive to the detrimental effects of calcium than are adult hearts (28).

There is tremendous value to lengthening the window of cardiac preservation. First, less ischemia to the donor organ will likely improve posttransplant graft function and recipient survival. Second, lengthening the window of cardiac preservation will allow prospective HLA matching as well as the transport of hearts over greater distances to better matched recipients.

Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20^0C yields left ventricular function that is superior to 8-hr perfusion with a chemically similar crystalloid solution without addition of PEG-Hb, despite similar myocardial edema. This study addresses myocardial performance following perfusion with and without the PEG-hemoglobin oxygen carrier, since the control group does not really represent an alternate myocardial preservation scheme. Similarly, the mechanism of preservation using this PEG-Hb solution may or may not involve enhanced oxygen delivery. Extended cardiac perfusion preservation with this PEG-Hb based solution deserves further study, including comparison to traditional cardioplegic preservation solutions. It is possible that PEG-Hb may be a useful component of a future clinical preservation solution.

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LEGEND

Figure 1. Isolated heart perfusion preservation circuit.

Figure 2. Developed LV pressure at 15, 75, and 135 minutes after preservation. The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant (*).

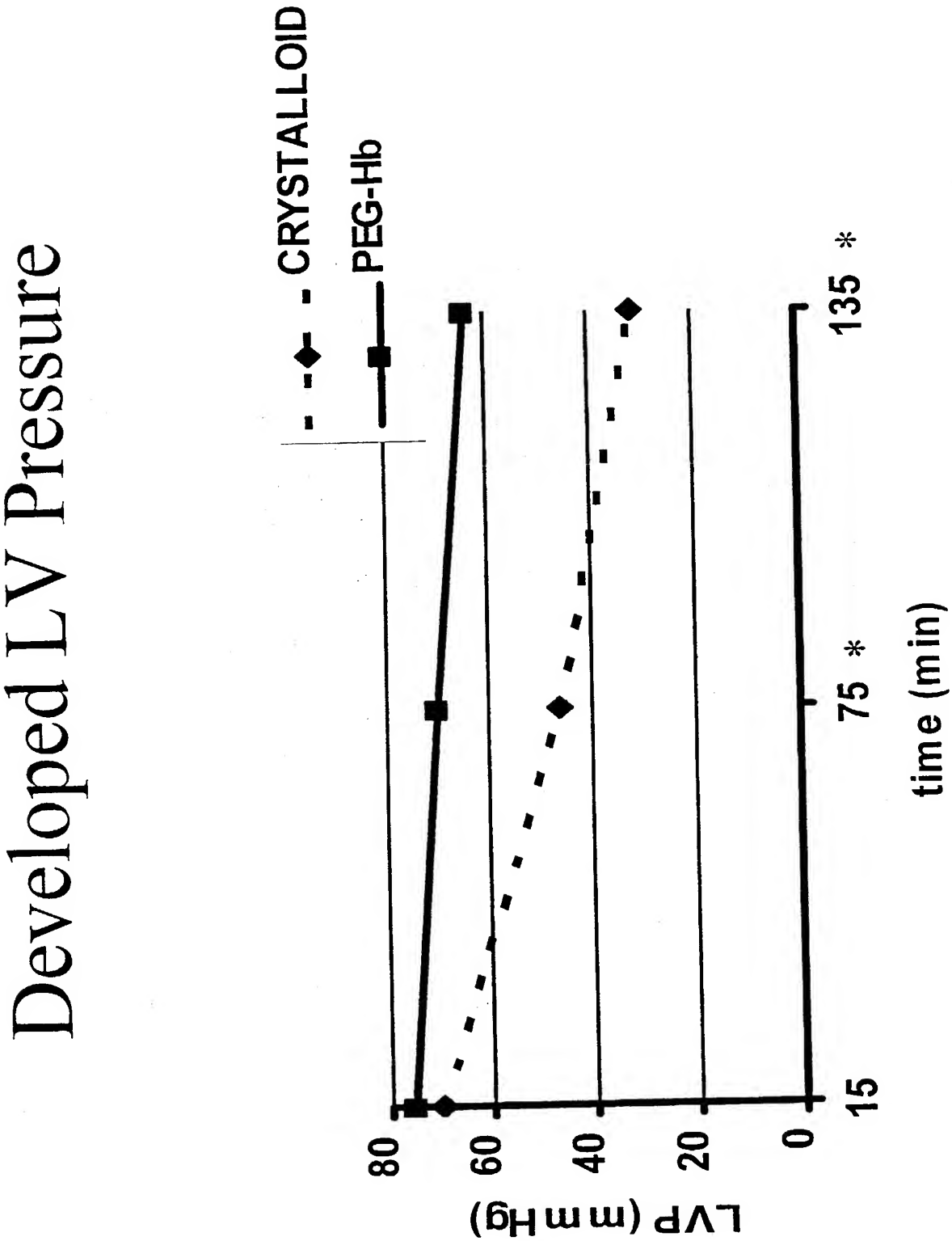
Figure 3. Maximum rate of LV contraction at 15, 75, and 135 minutes after preservation. The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant (*).

Figure 4. Maximum rate of LV relaxation at 15, 75, and 135 minutes after preservation. The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant (*).

Table 1. Heart rate after 8 hours of perfusion preservation.

	Heart Rate		
Time (minutes)	PEG-Hb	Crystalloid	p value
15	117±8.4	100.8±8.4	0.19
30	104.9±7.9	98.4±4.9	0.48
45	98.8±6.6	95.2±4.6	0.66
60	94.1±6.6	97.6±5.7	0.70
75	90.9±6.8	96.2±5.5	0.55
90	99.2±6.6	97.0±3.5	0.77
105	97.8±6.3	89.1±4.8	0.30
120	95.0±6.4	75.8±11.5	0.14
135	100.5±6.3	73.9±13.7	0.07

Table 1.


Developed LV Pressure
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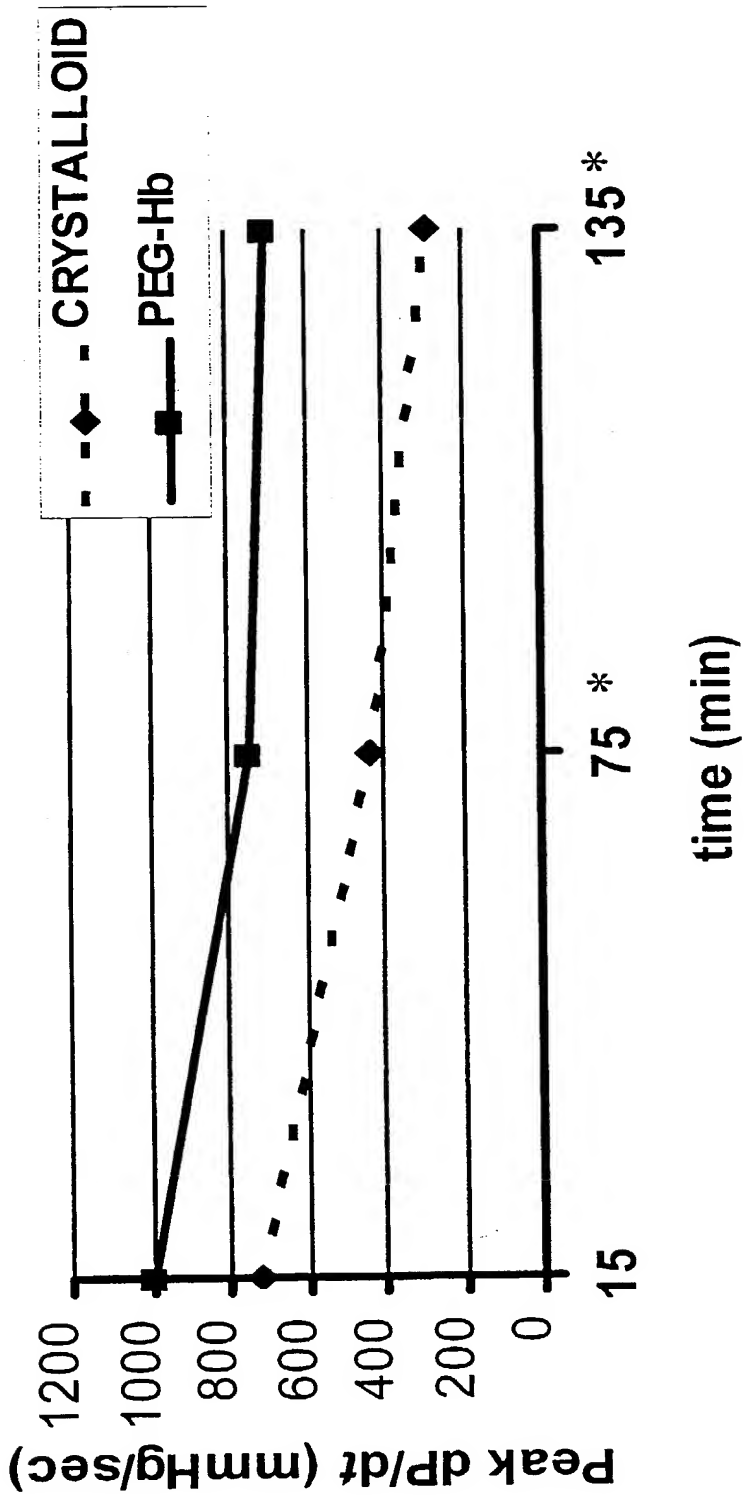
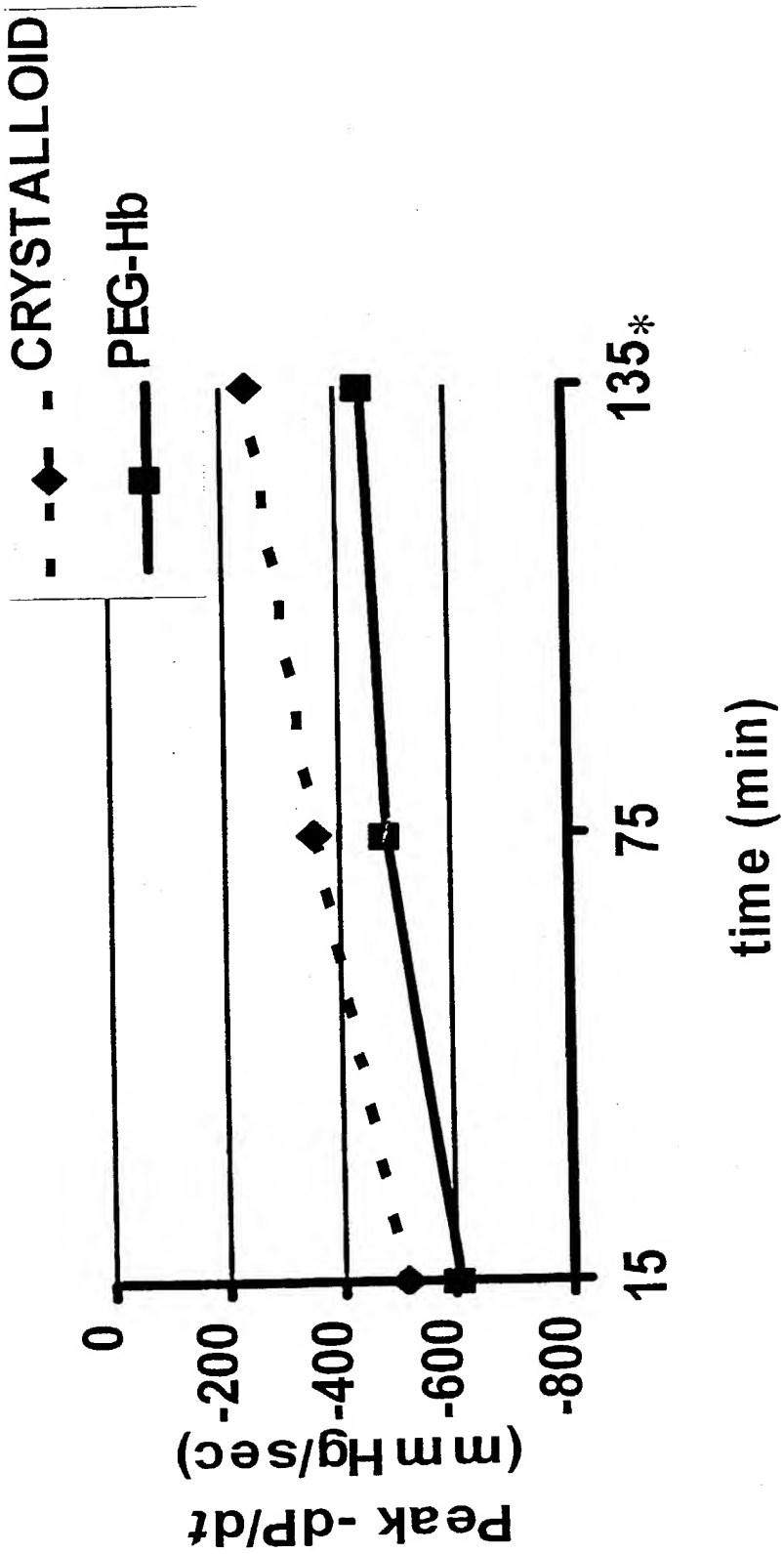
60
40
20
0
15
75 *
135 *
time (min)

Peak dP/dt


CRYSTALLOID
PEG-Hb
Peak dP/dt (mmHg/sec)
1200
1000
800
600
400
200
0
15
75 *
135 *
time (min)

Peak -dP/dt


CRYSTALLOID
PEG-Hb
Peak -dP/dt
(mmHg/sec)
0
-200
-400
-600
-800
15
75
135*
time (min)

APPENDIX 4

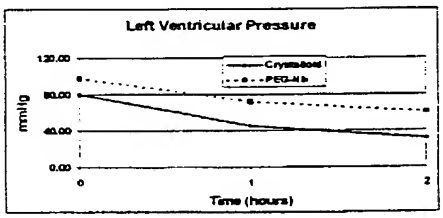
CARDIAC FUNCTION AFTER 8-HOUR PERFUSION PRESERVATION USING PEG-HEMOGLOBIN

Introduction: Efforts to extend myocardial preservation for transplantation by crystalloid perfusion has been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with an oxygen carrying hemoglobin solution may extend preservation times.

Preclinical Study 1:
The purpose of this study was to compare cardiac function after continuous perfusion with PEG-Hemoglobin (Hb) vs. a physiologic crystalloid perfusate.

Methods: The hearts of 9 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=4) hearts were continuously perfused with PEG-Hb (Enzon Inc., N.J.) at 20°C and 30 mmHg for 8 hours. Group II (n=5) hearts were continuously perfused with crystalloid perfusion for 8 hours at 20°C and 30 mmHg. To both solutions were added KCl (4 mmol/L), Na^+ (145 mmol/L), $MgSO_4$ (5.1 mmol/L), $CaCl_2$ (0.4 mmol/L), 12.5 mg/L lidocaine, heparin (1250 units/L), dextrose (1.25 gm/L), human albumin (1.6 gm/L), human insulin (3.1 units/L). PO_2 was maintained greater than 500 mmHg, and pH of 7.1 (37°C). Cardiac function was measured with a left ventricular balloon at 0, 1, and 2 hours after transfer to a standard crystalloid Langendorff circuit.

Results: Heart rate was the same for group I and II through the testing period (89.6 vs. 91.1, p=0.57). Developed LV pressure (systolic minus diastolic) at 0.6cc LV volume was greater in Group I (76.17±19.2 mmHg), than in Group II (52.0±25.21, p=0.021). Maximum +dP/dT at 0.6 cc LV volume was greater in Group I (854.47±381.8mmHg/sec) than in Group II (485.10±284.14 mmHg/sec, p=0.025). Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II.



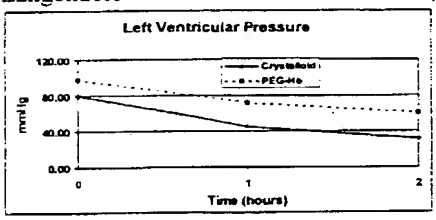
Attachment 2

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20°C yields left ventricular function is superior to 8-hr crystalloid perfusion, despite similar myocardial edema. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation.

Preclinical Study 2:
Methods: The hearts of 14 anesthetized and intubated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=4) hearts were perfused with PEG-Hb (Enzon Inc., N.J.) at 20°C and 30 mmHg for 8 hours. Group II (n=5) hearts were preserved without perfusion for 4 hours at 4°C, and Group III (n=5) were tested immediately upon harvest.

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING P HEMOGLOBIN VS. CRYSTALLOID PERFUSION

Efforts to extend myocardial preservation for transplantation crystalloid perfusion has been limited by edema and compromised funct We hypothesized that hypothermic perfusion preservation with an oxy carrying hemoglobin solution may extend preservation times. The purp of this study was to compare cardiac function after continuous perfu with PEG-Hemoglobin (Hb) vs. a physiologic crystalloid perfusate. hearts of 9 anesthetized and ventilated NZW rabbits were harvested a cold cardioplegic arrest. Group I (n=4) hearts were continuously perfi with PEG-Hb (Enzon Inc., N.J.) at 20°C and 30 mmHg for 8 hours. Gr II (n=5) hearts were continuously perfused with crystalloid perfusion f hours at 20°C. Cardiac function was measured with a left ventric balloon at 0, 1, and 2 hours after transfer to a standard crystal Langendorff circuit.

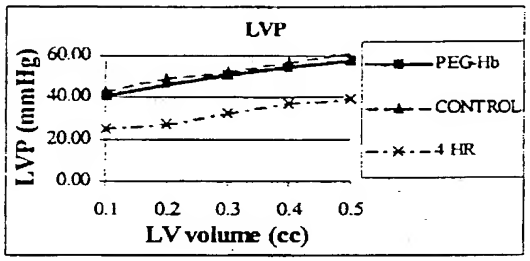


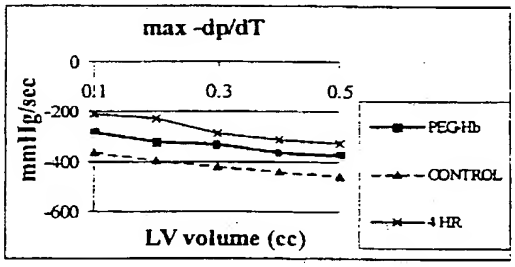
Heart rate was the same group I and II through testing period (89.6 vs. 91 p=0.57). Developed pressure (systolic mi diastolic) at 0.6cc LV volu was greater in Group (76.17±19.2 mmHg), than in Group II (52.0±25.21, p=0.021). Maximum +dP/dT at 0.6 cc LV volu was greater in Group I (854.47±381.8mmHg/sec) than in Group (485.10±284.14 mmHg/sec, p=0.025). Percent water of total ventric weight was 82.0% for Group I, 81.6% for Group II. Continuous perfu preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 2 yields left ventricular function is superior to 8-hr crystalloid perfus despite similar myocardial edema. Extended cardiac perfusion preserva with PEG-Hb may prove to be useful in transplantation.

DL Serna, LL Powell, WC Wallace, J West, C Kahwaji, G Cogen Smulowitz, E Steward, R Purdy, JC Milliken. UC Irvine.

To PEG-Hb solution was added KCl (4 mmol/L), Na^+ (145 mmol/L), $MgSO_4$ (5.1 mmol/L), $CaCl_2$ (0.4 mmol/L), 12.5 mg/L lidocaine, heparin (1250 units/L), dextrose (1.25gm/L), human albumin (1.6 gm/L), human insulin (3.1 units/L). PO_2 was maintained greater than 500 mmHg, and pH of 7.1 (37°C). Cardiac function was measured in the non-working state 2 hours after transfer to a standard crystalloid Langendorff circuit.

Results: Developed LV pressure at 0.5cc LV volume was similar in Group I (57.94±4.69 mmHg), and Group III (61.45±14.45 mmHg, p=0.71), but trended toward superiority compared to Group II (39.72±6.98 mmHg, p=0.32). Maximum –dp/dT at 0.5 cc LV volume was similar in Group I (–376.58±33.78 mmHg/sec), Group II (426.32±139.15 mmHg/sec, p=0.77), and Group III (–326.73±47.75 mmHg/sec, p=0.23). Percent water of total ventricular weight was 82.0% for Group I, 82.3% for Group II, and 81.5% for Group III.





Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20°C yields left ventricular function that may be superior to 4 hrs of hypothermic storage and is similar to that of non-ischemic control hearts. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation.

Continuous Cardiac Perfusion Preservation with PEG-Hb Improves Perfomance over Standard Ischemic Hypothermic Storage

Dan L. Serna, Ledford L. Powell, Chadi I. Kahwaji, William C. Wallace, Peter Smulowitz, Justin West, Peter Connolly, Gerry Beckham, Earl Steward, Ralph Purdy, Jeffrey C. Milliken.

Cardiac preservation for transplantation is generally limited by ischemic hypothermic storage of 4 to 6 hours. Hypothermic perfusion preservation with an oxygen carrying hemoglobin solution may extend preservation times and decrease ischemic injury. The purpose of this study was to compare cardiac function after 8 hrs of continuous hypothermic perfusion with a PEG-Hemoglobin(Hb) solution to the clinical standard of hypothermic ischemic preservation.

Methods: The hearts of 28 anesthetized and intubuted NZW rabbits were harvested after cold cardioplegic arrest. Group I(n=10) hearts were perfused with a PEG-Hb (Enzon, Inc., Piscataway, NJ) solution at 20C and 30 mmHg for 8 hours. Group II(n=10) hearts were preserved by cold ischemic storage for 4 hours at 4C, and Group III(n=8) were tested immediately after harvest. LV function was measured in the non-working state 15 min after transfer to a standard crystalloid Langendorff circuit.

Results: Developed LV pressure at 0.5cc LV volume was greater in Group I (75.7±10.3mmHg) than Group II (49.1±5.4mmHg,p=.04) and similar to Group III (69.41±5.1mmHg, p=.6). Maximum-dp/dT at 0.5 cc LV volume was greater in Group I(-610.6±68.4mmHg/sec) than Group II (-354.8±49.1mmHg/sec,p=.01) and trended toward superiority over Group III(-456.2±44.1mmHg/sec,p=.09). Maximum +dp/dT at 0.5cc LV volume was greater in Group I (964.9±156.6mmHg/sec) than both Group II (428.4±54.9mmHg/sec,p=.004) and Group III(514.6±48.9mmHg/sec,p=.02).

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb solution at 30 mmHg and 20 C yields left ventricular function that is superior to 4 hrs of ischemic hypothermic storage and is similar to that of fresh control hearts. Extended cardiac perfusion preservation with PEG-Hb deserves further investigation in large animal transplant models.

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING PEG-HEMOGLOBIN VS. CRYSTALLOID PERFUSION

DL Serna, RE Purdy, JC Milliken. UC Irvine Medical Center, Orange, CA.

Corresponding Author:

Dan L. Serna, MD

UC Irvine Medical Center

Division of Cardiothoracic Surgery

101 The City Drive, Bldg. 53, Rm. 117

Orange, CA 92868

(714) 456-3634 – office

(714) 456-8870 – fax

dlserna@uci.edu - email

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ABSTRACT

Introduction: Efforts to extend myocardial preservation for transplantation by crystalloid perfusion have been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with a polyethylene glycol (PEG) conjugated hemoglobin solution may extend preservation times. The purpose of this study was to compare cardiac function after continuous perfusion using a hypocalcemic normokalemic crystalloid perfusate with and without the addition of PEG-Hemoglobin (Hb).

Methods: The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a hypocalcemic, normokalemic 3% PEG-Hb solution at 20°C and 30 mmHg for 8 hours. Group II (n=10) hearts were continuously perfused with chemically similar crystalloid solution without PEG-Hb for 8 hours under identical conditions to Group I hearts. Cardiac function was measured with a left ventricular force transducer after transfer to a standard crystalloid Langendorff circuit at 37 °C and an aortic root pressure of 59 mmHg.

Results: After 8 hours of perfusion preservation, heart rate was similar for groups I and II (p=NS). Coronary blood flow after preservation and during preservation was similar between PEG-Hb and crystalloid preserved hearts (p=NS). Left ventricular developed pressure, peak dP/d*t*, and peak -dP/d*t* were superior in hearts preserved with PEG-Hb.

Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hours with a hypocalcemic normokalemic PEG-Hb based solution at 30 mmHg and 20°C yields left ventricular function that is superior to crystalloid perfusion without the addition of PEG-Hb, despite similar myocardial edema and coronary flow. Extended cardiac perfusion preservation with this PEG-Hb based solution deserves further study.

INTRODUCTION

Heart failure affects more than 3 million patients in the United States (1). Almost one-third of these patients have New York Heart Association functional class III or IV heart failure, often characterized by progressive deterioration and frequent hospital admissions. Annual expenditures for heart failure have been estimated to be as high as $38 billion, of which $23 billion is for hospital stays and non-surgical treatment prior to transplantation (1). Federal legislation has recently been passed allowing the distribution of donor organs to recipient matches outside the geographic range of the donor. Meanwhile, existing techniques of preservation and donor organ distribution remain archaic. There is both a humanitarian and economic need to develop innovative techniques of donor heart procurement, preservation, and distribution.

The current method of donor heart preservation involves cold cardioplegic arrest and storage at near freezing temperatures. Because of ongoing ischemia, this preservation technique prohibits extended storage of donor organs, use of more efficacious methods of tissue typing, as well as delivery of donor hearts over large distances. The current preservation technique also leads to irreversible graft damage.

Preservation by continuous coronary artery perfusion allows for greater preservation times than hypothermic ischemic preservation (2). Continuous coronary artery perfusion allows for an ongoing supply of substrate as well as removal of metabolic waste products. Three general types of solution have been examined for their efficacy as cardiac preservation agents. Perfusion with crystalloid, cardioplegia-type solutions have shown limited promise (3-7). Perfusion preservation using these solutions has been limited by edema and compromised cardiac function (3-7). Similarly, studies examining perfluorocarbon emulsions as perfusion preservation media for the donor heart have produced mixed results (2,8-11). Further, perfluorochemicals are very expensive and have questionable safety profiles (8-10).

Hemoglobin-based blood substitutes have more recently been developed for use as blood replacements in trauma situations and surgery. Use of these solutions as organ preservation solutions may lengthen the window of *ex vivo* cardiac preservation with a concomitant decrease in ischemia. We hypothesized that hypothermic perfusion preservation with an hypocalcemic, normokalemic, polyethylene glycol conjugated hemoglobin (PEG-Hb) based solution over 8 hours would preserve left ventricular function above that obtained with a chemically identical crystalloid solution without PEG-Hb. We suspect that this PEG-Hb solution may optimize the donor heart during its preservation period. Moreover, optimization of perfusion preservation using this solution may, in time, allow considerable widening of the window of *ex vivo* cardiac preservation, allowing transportation of donor organs over large distances, more thorough tissue typing and matching, and as well as improved post-implantation graft function and survival.

The purpose of this study is to compare *ex vivo* adult rabbit heart preservation after continuous coronary artery perfusion using a hypocalcemic, normokalemic PEG-Hb solution versus an identical crystalloid solution not containing PEG-Hb. This work will lay the foundation for future investigation comparing perfusion preservation with PEG-Hb based solutions to hypothermic ischemic storage preservation as well as PEG-Hb solutions containing specific enhancers of myocardial preservation.

METHODS

All animals received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23). The Institutional Animal Care and Use Committee of the University of California, Irvine, approved animal procedures.

Experimental Design

The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a normokalemic hypocalcemic PEG-Hb based solution at 20°C and 30 mmHg of aortic root pressure for 8 hours. Group II (n=10) hearts were identically preserved with a crystalloid solution identical in composition to Group I hearts, but without the addition of PEG-Hb.

Cardiac Procurement

Twenty adult male New Zealand White rabbits (3 to 3.5 kg) were anesthetized using an intramuscular injection of 50mg ketamine and 5mg xylazine per kilogram. Lactated Ringers solution was infused through an IV catheter in a marginal ear vein at a rate of 5 to 15 cc/hr. The rabbits were mechanically ventilated using a Servo Animal

Ventilator (model #900C, Siemens-Elema, Sweden). Anesthesia was maintained with intravenous ketamine/xylazine in a 1:1 ratio. A median sternotomy followed by a longitudinal pericardial incision was performed, exposing the heart and mediastinal vessels. All rabbits received 1,000 U heparin sodium/kg IV. The innominate artery, the aortic arch between the brachiocephalic trunk and left carotid artery, as well as the inferior and superior vena cava were the identified and isolated. Upon ligation of the inferior and superior vena cava, the innominate artery was cannulated using an 18 Ga angiocatheter. 60 cc of hypothermic cardioplegia solution (2-4°C) was administered to the coronary arteries via the innominate artery over 3 minutes. An arteriotomy was made in the pulmonary trunk to decompress the right ventricle. Hypothermic normal saline (2-4°C) was used to cool the heart during cardioplegia infusion. The heart was quickly excised and placed in cold saline (4°C) for further dissection. The heart was trimmed of excess soft tissue including lungs, trachea, and thymus. All hearts were placed onto the preservation circuit by cannulation at the ascending aorta. Coronary perfusion was begun within 5 minutes of cardiectomy. All hearts were preserved for 8 hours by continuous coronary artery perfusion. Aortic root pressure was maintained at 30 mmHg. Temperature of the perfusate was maintained at 20°C. All hearts were perfused and immersed in the respective preservation solutions for the entire 8-hour preservation period. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to the preservation circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. The preservation circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus

Inc., Eden Prairie, MN), an adult membrane oxygenator (Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA) which was circulated through the membrane oxygenator (figure 1).

Preservation Solutions

The composition of the PEG-Hb based preservation fluids is as follows: 3% PEG-Hb, KCL (4.7 mEq/L), NaCl (148.7 mmol/L), NaPO4 (2.5 mmol/L), NaHCO3 (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), Tromethamine (THAM) solution (7.3 cc/L).

The composition of the crystalloid preservation solution is as follows: KCL (4.7 mEq/L), NaCl (150.7 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6 units/L), and Tromethamine (THAM) solution (7.3 cc/L).

Postpreservation Assessment of Cardiac Function:

At the end of the 8-hour preservation period, all hearts were transferred to an isolated heart perfusion apparatus for purposes of data collection. Coronary perfusion via the aortic root was immediately begun at 37°C and 59 mmHg aortic root pressure. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to this circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. After 15 minutes of coronary perfusion in this position, coronary flow, heart rate, left ventricular developed pressure (LVP), peak dP/d*t*, and peak –dP/d*t* were

measured. Coronary flow and heart rate were measured every 15 minutes for 2 hours. LVP, peak dP/d*t*, and peak –dP/d*t* were measured again at 75 and 135 minutes following transfer to the second circuit. Heart rate was measured by counting left ventricular contractions over the course of one minute. Coronary flow was measured by collecting the effluent that exited from the pulmonary artery over course of one minute. LVP, peak dP/d*t*, and peak –dP/d*t* were measured in the beating, nonworking position during continuous coronary artery perfusion. Developed left ventricular pressure (systolic minus diastolic) and peak rates of left ventricular pressure development (dP/dt_{max}) and relaxation ($-dP/dt_{max}$) were measured using a left ventricular force transducer (Biopac Systems, Inc., Santa Barbara, CA). Data from the LV force transducer was digitized using an analog to digital converter (Biopac Systems, Inc., Santa Barbara, CA) and analyzed using Acknowledge software (Version 3.2.6, Biopac Systems, Inc., Santa Barbara, CA) and a desktop computer (Nexstar, Fremont, CA). The testing circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus Inc., Eden Prairie, MN), an adult membrane oxygenator (Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA) which was circulated through the membrane oxygenator.

Testing Solution: Left ventricular function was assessed in all hearts in both groups using a standard physiologic crystalloid solution. The composition was as follows: KCL

(4.7 mEq/L), NaCl (151.5 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (2.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human insulin (30.7 units/L), and Tromethamine (THAM) solution (6.1 cc/L).

Measurement of ventricular water content: After 135 minutes of retrograde aortic perfusion on the testing circuit, the ventricular myocardium of the initial 5 hearts in each group was dissected free of atria and other soft tissue. The left ventricular myocardium was weighed before and after desiccation at 110°C.

Data Analysis

Data are reported as means ± SE. Statistical analysis was performed using Systal 7.0.1 software package (SPSS, Inc., Chicago, IL). A p value of less than 0.05 was considered significant.

Materials

Bovine PEG-Hb was obtained from Enzon, Inc. (Piscataway, NJ) in a solution containing 6% PEG-Hb, 5 mM NaPO4, 5 mM NaHCO3, and 150 mM NaCl. Normal saline solution (0.9% NaCl) was obtained from Baxter Health Care (Irvine, CA). Solutions were monitored using a blood gas analyzer (288 Blood Gas System, Ciba-Corning Diagnostics Corp., Medfield, MA), an Automated Coagulation Timer (Medtronic Hemotec, Inc., Englewood, CO) and a blood glucose meter (Lifescan, Inc., Milpitas, CA). Membrane oxygenators were obtained from Sarns/3M Health Care, Inc. (Ann Arbor, MI).

RESULTS

Developed LV pressure: Developed LV pressure at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes after the end of

preservation (p=0.46, figure 2). However, developed LV pressure at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 (p=0.006) and 135 minutes (p=0.002) after the end of preservation.

Maximum rate of LV contraction: Peak dP/dt_{max} at 0.5 cc LV volume trended toward superiority amongst hearts preserved using PEG-Hb solution compared to crystalloid preserved hearts, at 15 minutes after the end of preservation (p=0.10, figure 3). However, peak dP/dt_{max} at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 (p=0.01) and 135 minutes (p=0.001) after the end of preservation.

Maximum rate of LV relaxation: Peak - dP/dt_{max} at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes (p=0.27) after the end of preservation and trended toward superiority at 75 minutes after the end of preservation (p=0.07, figure 4). Peak - dP/dt_{max} at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 135 minutes (p=0.006) after the end of preservation.

Ventricular Water Content: Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Coronary Flow: Coronary flow after preservation was similar between PEG-Hb and crystalloid preserved hearts (figure 5).

Heart Rate: Heart rate was the same for group I and II through the testing period (p=NS, Table 1).

CONCLUSIONS

There are two general techniques of *ex vivo* cardiac preservation for transplantation. The standard of care and currently used technique is hypothermic ischemic immersion storage of the donor cardiac allograft. The second method of cardiac preservation is coronary perfusion preservation. These two methods can and have been used in combination with improved results. Perfusion preservation is superior to immersion preservation as it allows the continuous washout of metabolic waste products, as well as delivery of nutrients, metabolic substrate, and depending upon the solution used, oxygen to the myocardium (2). For several reasons, perfusion preservation has not been applied clinically to *ex vivo* cardiac preservation for transplantation. First, a user friendly, practical, and portable perfusion preservation device is not currently available. Second, most research into perfusion preservation to date has been performed using saline-based cardioplegia solutions and perfluorocarbons. Saline-based cardioplegia solutions, unfortunately, carry very little oxygen and hence their use is associated with considerable ischemic injury to the donor organ (3-7). Perfluorocarbon based solutions have demonstrated mixed results for the purpose of cardiac preservation, are extremely expensive and possibly toxic (2,8-11).

Perfusion preservation using stroma-free hemoglobin based solutions represents an innovative means of *ex vivo* cardiac preservation. Stroma-free hemoglobins were initially developed as blood substitutes for use in the treatment of life threatening hemorrhage secondary to trauma. There is strong interest amongst transplant scientists in the potential for these solutions as organ preservation solutions. The purpose of this

study was to assess the utility of perfusion preservation using normokalemic hypocalcemic polyethylene glycol coated bovine hemoglobin based solution.

The covalent attachment of PEG to the Hb gives the Hb entity a bigger particle size, which may help to maintain the hemoglobin within the vascular space by making it too large to easily migrate into the interstitial space. One question that arises is whether the mechanism of benefit in this study is secondary to the PEG or the hemoglobin or the combination of PEG with hemoblobin? There exists theoretical basis for a beneficial action by both PEG as well as hemoglobin when each is used alone. When added to Bretschneider's HTK cardioplegic solution, PEG is associated with improved recovery of left ventricular function as well as less myocardial edema (12). The mechanism of action of PEG is uncertain, but probably includes prevention of osmotic swelling and suppression of lipid peroxidation (12). Hemoglobin, meanwhile, is capable of carrying and delivering oxygen to the myocardiocytes. Similarly, hemoglobin likely contributes to the oncotic effect of the solution, acting to minimize tissue edema.

We made the preservation solution hypocalcemic because the intracellular accumulation of calcium during ischemia and reperfusion is associated with cellular injury (13-17) and a hypoxically stressed heart may be protected by a hypocalcemic solution (13). We made the solution normokalemic since we suspected that a beating heart would be less susceptible to edema formation in the setting of adequate oxygen delivery. Finally, we made the preservation solution slightly hypermagnesemic because magnesium inhibits the membrane transport of calcium, and thus intracellular accumulation of calcium, which should help to prevent the deleterious effects of calcium (18-21). Magnesium has been shown to attenuate deleterious effects of calcium in

ischemic piglet hearts, which are more sensitive to the detrimental effects of calcium than are adult hearts (22).

This study utilized bovine hemoglobin, which is similar in structure to human hemoglobin but possess several important differences. First, the oxygen-hemoglobin dissociation curve is slightly shifted such that bovine hemoglobin more easily releases oxygen at the tissue level. Second, while 2,3-diphosphoglycerate decreases the affinity of human hemoglobin for oxygen, chloride anion similarly binds to and affects the affinity of bovine hemoglobin for oxygen, increasing the dissociation of oxygen from bovine hemoglobin at the tissue level. PEG-modified Hb, in a formulation different from that described in this manuscript, is currently being investigated in phase II clinical trials for use as an enhancer to radiation therapy in patients with cancer.

There is tremendous value to lengthening the window of cardiac preservation. First, less ischemia to the donor organ improves posttransplant graft function and recipient survival. Second, lengthening the window of cardiac preservation will allow prospective HLA matching as well as the transport of hearts over greater distances to better matched recipients. HLA mismatching results in an increased risk of early high-grade rejection. This results in rehospitalization and an increased use of resources. Coronary artery vasculopathy (CAV) is related to the degree of HLA mismatching. There is increased CAV among patients with more rejection episodes (23). HLA-DR mismatching has been shown to have strong adverse effects on graft survival when examined for up to 10 years (24-25).

This solution should be tested in the preservation of other organs as well. The heart is the most metabolically active and the most sensitive to ischemia of the

commonly transplanted solid organs. This data suggests PEG-Hb based solutions should be useful is the perfusion preservation of other solid transplantable organs. This suspicion should be investigated with controlled animal experiments. Future investigation will also be useful in the optimization of these Hb based solutions. Some questions that remain include the optimal preservation temperature, Hb concentration, as well as the optimal potassium concentration.

Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20^{0}C yields left ventricular function that is superior to 8-hr perfusion with a chemically similar crystalloid solution without addition of PEG-Hb, despite similar myocardial edema. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation. The next line of investigation should compare perfusion preservation with fresh hearts and with hearts that have been preserved using techniques similar to the standard of care. Furthermore, preserved hearts should be transplanted to evaluate left ventricular function after reperfusion with blood.

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LEGEND

Figure 1. Isolated heart perfusion preservation circuit.

Figure 2. Developed LV pressure at 15, 75, and 135 minutes after preservation.

Figure 3. Maximum rate of LV contraction at 15, 75, and 135 minutes after preservation.

Figure 4. Maximum rate of LV relaxation at 15, 75, and 135 minutes after preservation.

Figure 5. Coronary blood flow after 8 hours of perfusion preservation.

Table 1. Heart rate after 8 hours of perfusion preservation.

	Heart Rate		
Time (minutes)	PEG-Hb	Crystalloid	p value
15	117±8.4	100.8±8.4	0.19
30	104.9±7.9	98.4±4.9	0.48
45	98.8±6.6	95.2±4.6	0.66
60	94.1±6.6	97.6±5.7	0.70
75	90.9±6.8	96.2±5.5	0.55
90	99.2±6.6	97.0±3.5	0.77
105	97.8±6.3	89.1±4.8	0.30
120	95.0±6.4	75.8±11.5	0.14
135	100.5±6.3	73.9±13.7	0.07

Table 1.

AMENDED CLAIMS

[received by the International Bureau on 20 November 2000 (20.11.00);
original claims 1-3 replaced by new claims 1-30 (6 pages)]

1. A composition for donor organ preservation for transplantation comprising a crystalloid based solution of constituents including PEG-Hb, one or more physiologically essential electrolyte, at least one soluble protein, at least one nutritional formulation, and at least one agent acting on the cardiovascular system.

2. The composition of claim 1 where said electrolyte comprises $MgSO_4$, KCl, $CaCl_2$, NaCl, $NaHCO_3$, and Na_2HP0_4, NaH_2P0_4 or both.

3. The composition of claim 1 where said at least one soluble protein comprises human albumin.

4. The composition of claim 1 where said at least one soluble protein comprises human insulin.

5. The composition of claim 1 where said at least one nutritional formulation comprises a simple sugar.

6. The composition of claim 5 where said simple sugar comprises dextrose.

7. The composition of claim 1 where said at least one nutritional formulation comprises a carbohydrate and its metabolites.

8. The composition of claim 1 where said at least one nutritional formulation comprises an antioxidant.

9. The composition of claim 8 where said antioxidant is at least one selected from the group comprising glutathione, lipoic acid, N-acetyl cysteine, tocopherols, ascorbic acid, L-thiazolidine-2-one-4-carboxylic acid.

10. The composition of claim 1 where said at least one agent acting on the cardiovascular system comprises heparin sodium.

11. The composition of claim 1 where said at least one agent acting on the cardiovascular system comprises lidocaine HCl.

12. The composition of claim 1 where said crystalloid based solution comprises approximately 3% PEG-Hb by volume.

13. The composition of claim 1 where at least one of the constituents comprises one selected from the group of KCl (4.7 mEq/L), NaCl (148.7 mmol/L), Na_2HPO_4/NaH_2PO_4 (2.5 mmol/L), $NaHCO_3$ (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), 0.3M tromethamine (THAM) solution (7.3 cc/L).

14. The composition of claim 1 wherein said crystalloid based solution has a pH maintained at 7.1 as measured at 37°C.

15. The composition of claim 1 wherein crystalloid based solution has a pH maintained at 7.4 as measured at 20°C.

16. The composition of claim 1 where crystalloid based solution is used for *ex vivo* preservation of donor organ allografts during transportation for the purpose of transplantation.

17. The composition of claim 1 where said crystalloid based solution is used for in vivo myocardial preservation during open-heart surgery.

18. The composition of claim 1 where said crystalloid based solution is used as a blood substitute or blood replacement.

19. The composition of claim 1 where said crystalloid based solution is modified to increase the potassium concentration to reflect intracellular levels for the purposes of achieving cardioplegia or hypothermic cardiac arrest.

20. A composition for donor organ preservation for transplantation comprising a polyethylene glycol substituted bovine hemoglobin based solution for the purpose of ex vivo donor organ preservation to preserve donor human and animal organs, *ex vivo*, prior to transplantation.

21. The composition for donor organ preservation of claim 20 where said polyethylene glycol substituted bovine hemoglobin based solution comprises PEG-Hb, and at least one of the constituents selected from the group of human albumin, dextrose, heparin sodium, lidocaine HCl, $MgSO_4$, KCl, $CaCl_2$, 0.3M tromethamine (THAM) solution, NaCl, $NaHCO_3$, and Na_2HP0_4/NaH_2P0_4.

22. The composition for donor organ preservation of claim 21 where said polyethylene glycol substituted bovine hemoglobin based solution comprises approximately 3% PEG-Hb by volume.

23. The composition of claim 22 where at least one of the constituents comprises one selected from the group of KCl (4.7 mEq/L), NaCl (148.7 mmol/L), NaH_2PO_4/Na_2HPO_4 (2.5 mmol/L), NaHCO3 (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), 0.3M tromethamine (THAM) solution (7.3 cc/L).

24. A composition for donor organ preservation for transplantation of a donor organ comprising an oxygen, nutritional and electrolyte environment for tissue of said donor organ to provide *ex vivo* preservation such that said donor organ regains acceptable function post transplantation.

25. The composition for donor organ preservation of claim 24 where said oxygen, nutritional and electrolyte environment comprises PEG-Hb, and at least one of the constituents selected from the group of human albumin, dextrose, heparin sodium,

lidocaine HCl, $MgSO_4$, KCl, $CaCl_2$, 0.3M tromethamine (THAM) solution, NaCl, $NaHCO_3$, and Na_2HPO_4/NaH_2PO_4.

26. The composition for donor organ preservation of claim 24 where said oxygen, nutritional and electrolyte environment comprises approximately 3% PEG-Hb by volume.

27. The composition of claim 24 where at least one of the constituents comprises one selected from the group of KCl (4.7 mEq/L), NaCl (148.7 mmol/L), Na_2HPO_4/NaH_2PO_4 (2.5 mmol/L), $NaHCO_3$ (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), 0.3M tromethamine (THAM) solution (7.3 cc/L).

28. A method for harvesting donor organs comprising:

excising said donor organ;

perfusing said donor organ with a normokalemic hypocalcemic bovine PEG-Hb based solution; and

preserving said donor organ at a temperature for a predetermined time while continuing perfusion with said normokalemic hypocalcemic bovine PEG-Hb based solution in an oxygenated environment.

29. The method of claim 28 where preserving said donor organ in an oxygenated environment comprises oxygenating said normokalemic hypocalcemic bovine PEG-Hb based solution with $95\%O_2/5\%CO_2$.

30. The method of claim 28 where perfusing said donor organ with a normokalemic hypocalcemic bovine PEG-Hb based solution comprises continuously perfusing PEG-Hb, and at least one of the constituents selected from the group of human albumin, dextrose, heparin sodium, lidocaine HCl, $MgSO_4$, KCl, $CaCl_2$, Tromethamine (THAM) solution, NaCl, $NaHCO_3$, and Na_2HP0_4/NaH_2P0_4.

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(71) Applicant *(for all designated States except US)*: **REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 5th Floor, Oakland, CA 94607-5200 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SERNA Danny, L. Jr.** [US/US]; -. **MILLIKEN, Jeffrey, C.** [US/US]; -. **PURDY, Ralph, E.** [US/US]; -.

(74) Agent: **DAWES, Daniel, L.**; Myers, Dawes & Andras LLP, 650 Town Center Drive, Suite 650, Costa Mesa, CA 92626 (US).

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— *With amended claims.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CONTINUOUS CARDIAC PERFUSION PRESERVATION WITH PEG-HB FOR IMPROVED HYPOTHERMIC STORAGE

(57) Abstract: The proposed use of the invention is for the *ex vivo* preservation of human and animal donor organ allografts during transportation from the donor to the recipient for the purpose of transplantation. In addition to its use for ex vivo myocardial preservation, this PEG-Hb solution has tremendous potential utility for in vivo myocardial preservation during open heart surgery as well as a blood substitute or blood replacement during or following surgery of any sort, including open heart surgery. The invention comprises a polyethylene glycol coated bovine hemoglobin based solution for the purpose of ex vivo donor organ preservation and the use of the same. The fundamental principle of the solution is to provide an oxygen, nutritional and electrolyte environment to the tissue of the donor organ that is conducive to *ex vivo* preservation such that the donor organ will regain acceptable function post transplantation. The solution provides oxygen, a carbohydrate energy source, continuous metabolite washout and continuous perfusion with an isotonic, normokalemic, hypocalcemic solution that drastically improves myocardial preservation over current techniques considered the standard of care. Donor organ preservation for transplantation is performed using ischemic hypothermic immersion storage in saline solution using hypothermic perfusion preservation with an oxygen carrying hemoglobin solution. The solution contains PEG-Hb, human albumin, dextrose, heparin sodium, lidocaine HC1, $MgSO_4$, KC1, $CaC1_2$, THAM, NaC1, NaHCO3, Na_3PO_4, without which PEG-Hb is lethal to the myocardium and cannot be used for the purpose of effective organ preservation.

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Figure 1

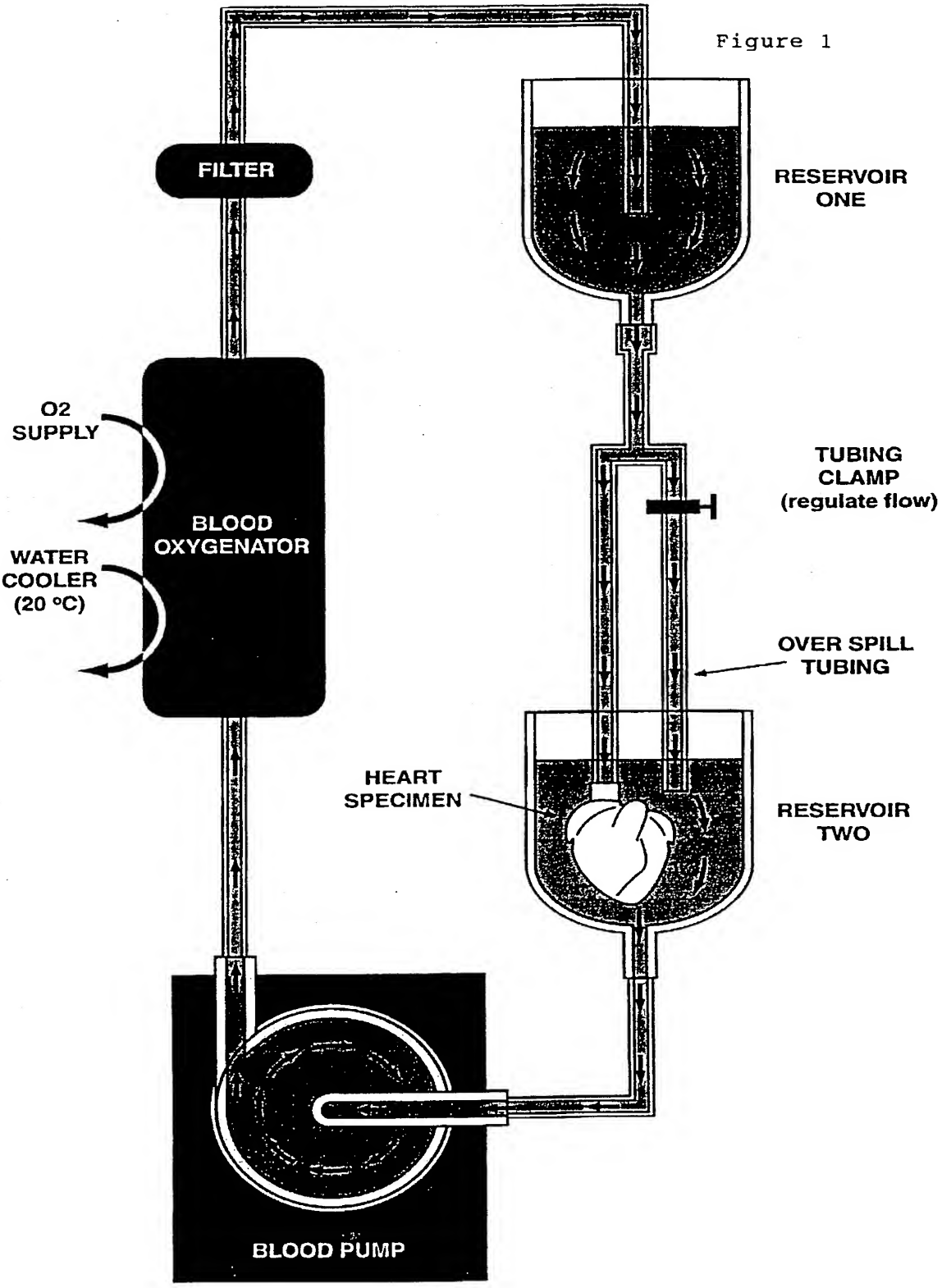

FILTER
RESERVOIR ONE
O2 SUPPLY
BLOOD OXYGENATOR
WATER COOLER (20 °C)
TUBING CLAMP (regulate flow)
OVER SPILL TUBING
HEART SPECIMEN
RESERVOIR TWO
BLOOD PUMP

Figure 2

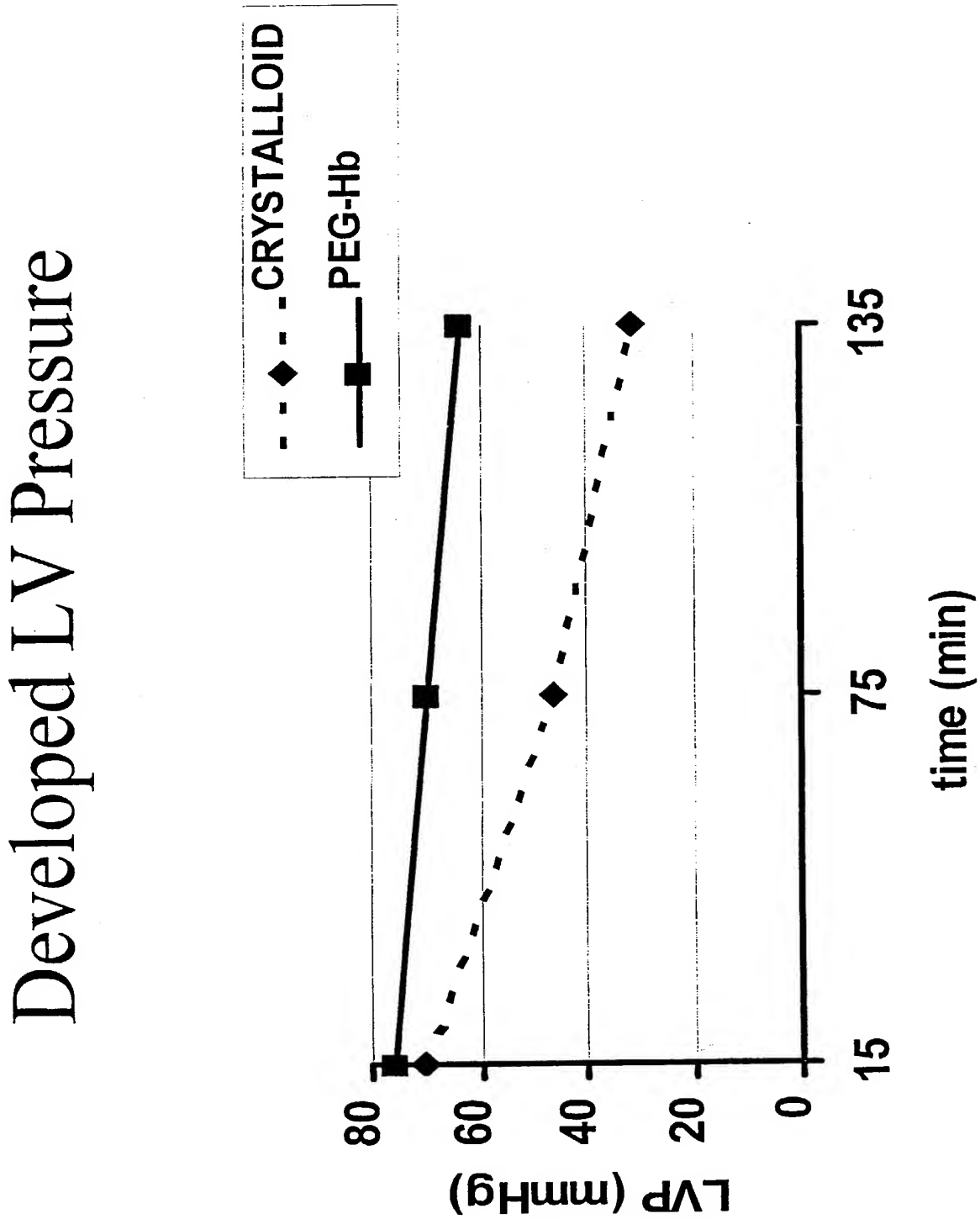

Developed LV Pressure
CRYSTALLOID
PEG-Hb
LVP (mmHg)
0
20
40
60
80
15
75
135
time (min)

Figure 3

Peak dP/dt

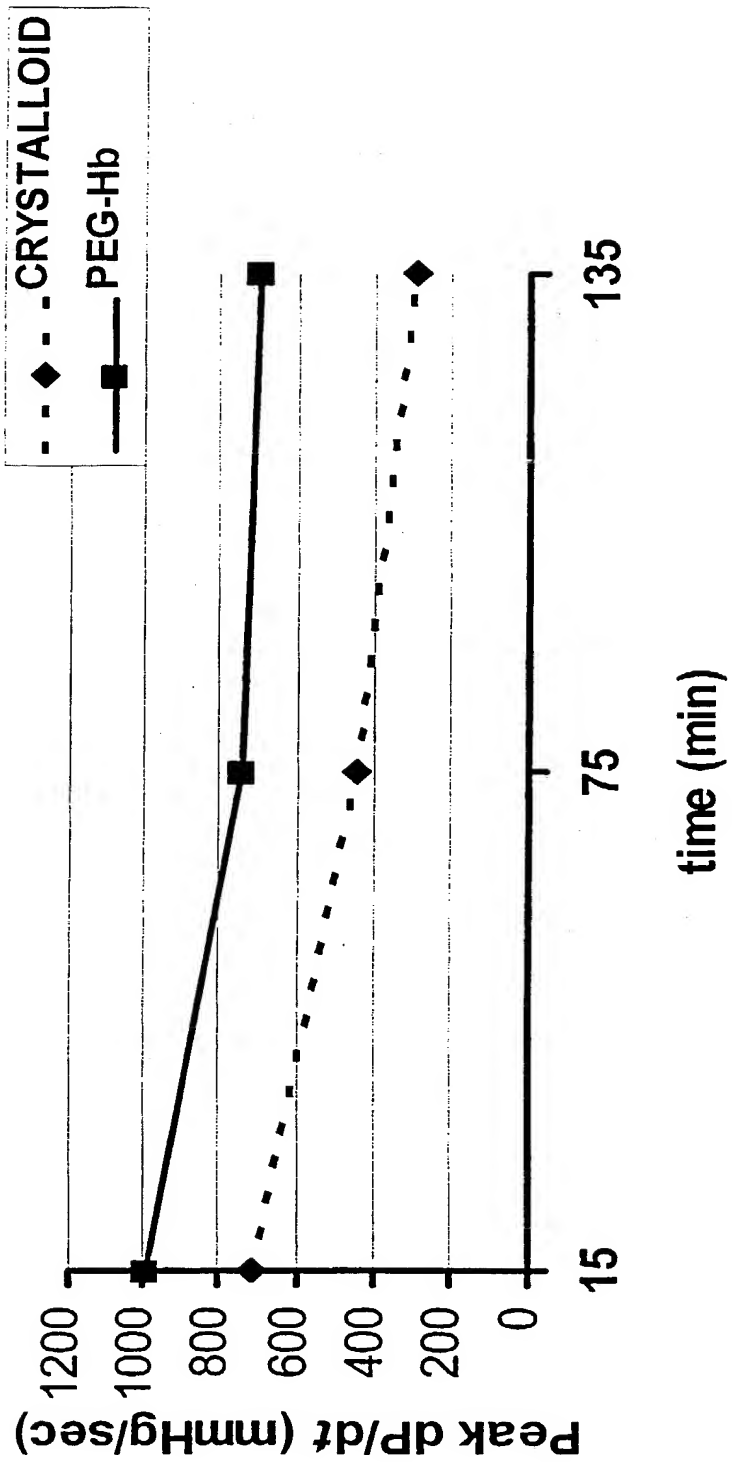


Figure 4

Peak -dP/dt

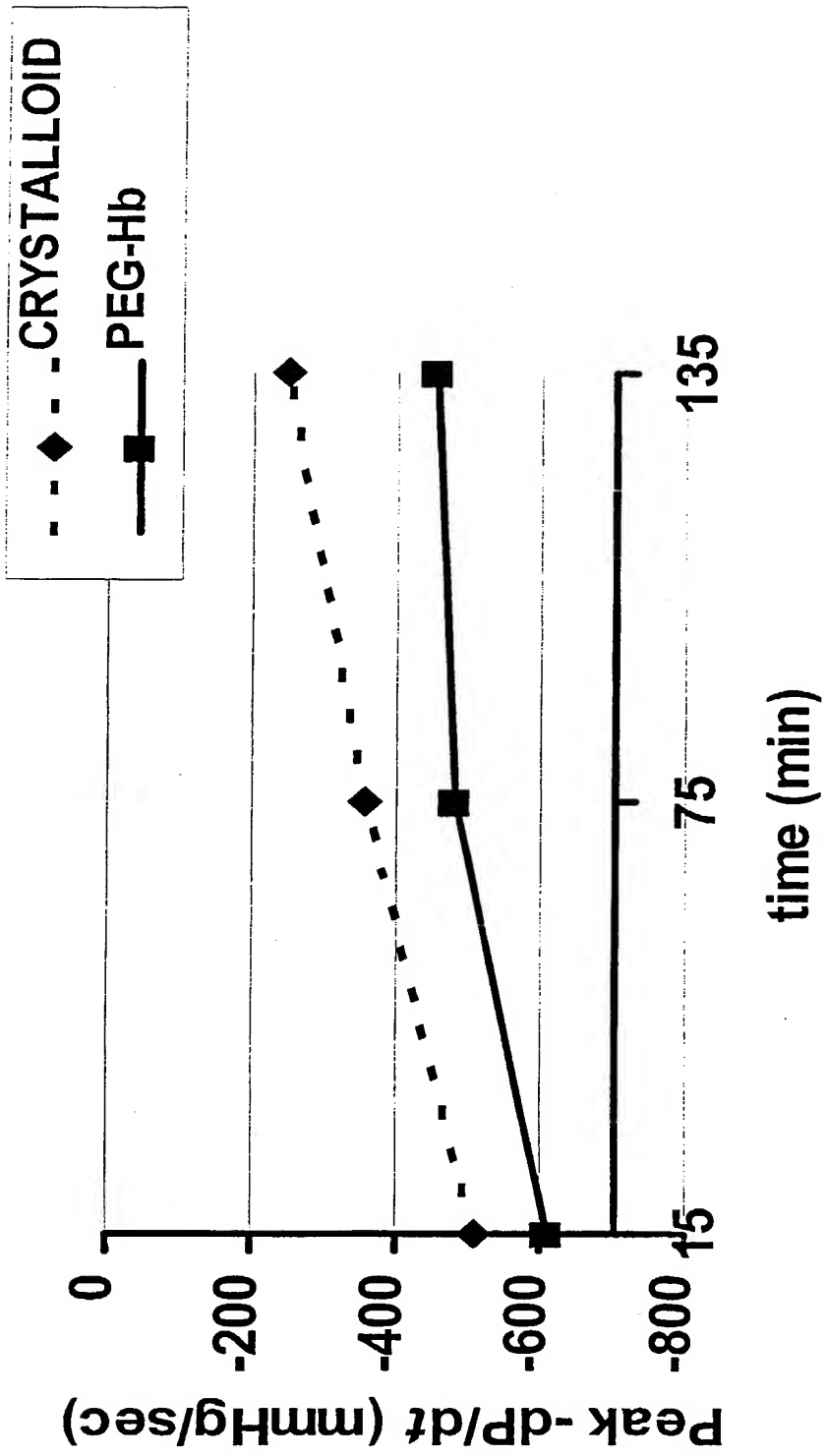
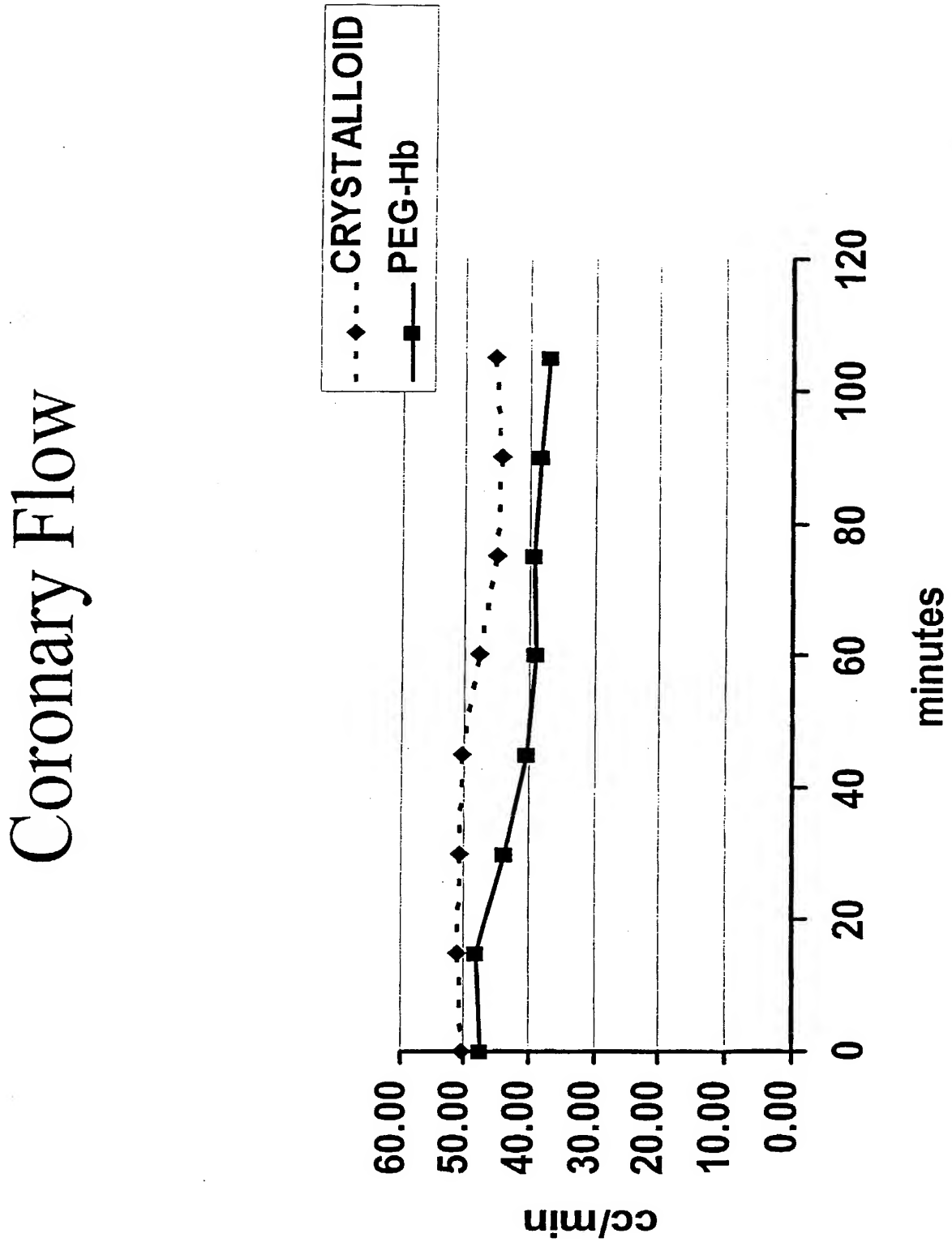


Figure 5


Coronary Flow
CRYSTALLOID
PEG-Hb
cc/min
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
20
40
60
80
100
120
minutes

DECLARATION OF INVENTORSHIP and

LIMITED POWER OF ATTORNEY

As a below named inventor, I believe that I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the invention entitled "Continuous Cardiac Perfusion Preservation with PEG-HB for Improved Ischemic Hypothermic Storage" which is described and claimed in Application No. 10/018,534 filed December 17, 2001 for which a patent is sought. My residence, post office address and citizenship are as stated below next to my name.

I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to herein.

I acknowledge my duty under Title 37, Code of Federal Regulations * 1.56(a) to disclose information which is material to the patentability of the invention I am claiming.

* I hereby claim the benefit under Title 35, United States Code, * 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code * 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, * 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application: [Parent serial no.], filed [Parent filing date], now [Parent status].

As a named inventor and until I assign my rights to the invention, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith on my behalf: **Frank J. Uxa**, Reg. No. 25,612; **Donald E. Stout**, Reg. No. 34,493; **Robert D. Buyan**, Reg. No. 32,460; **Kenton R. Mullins**, Reg. No. 36,331; **Jo Anne M. Ybaben,** Reg. No. 42,243; **Linda Allyson Fox**, Reg. No.38,883; **Kyle D. Yesland, Ph.D.**, Reg. No. 45,526; **Greg S. Hollrigel, Ph.D.**, Registered Patent Agent, Reg. No. 45,374; **Louise S. Heim**, Registered Patent Agent, Reg. No. 32,337, all of the firm STOUT, UXA, BUYAN & MULLINS, LLP,. **Send correspondence and direct telephone calls to: Robert D. Buyan, Stout, Uxa, Buyan & Mullins, LLP, 4 Venture, Suite 300, Irvine, CA 92618; telephone (949) 450-1750, facsimile (949) 450-1764, email: rbuyan@patlawyers.com**.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that there statements

were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application and any patent issuing thereon.

Danny L. Serna, of, Atlanta, Georgia, a citizen of the U.S.A. Post office address: 1012 Oak Park Circle, Atlanta, GA, 30324 GA

Signature: Date: 10/28/02

Danny L. Serna Danny L. Serna

Jeffrey C. Milliken, of, Newport Coast, California, a citizen of the U.S.A. Post office address: 14 Fairwind, Newport Coast, CA, 92687 CA

Signature: XXXXXXXX Date:

Jeffrey C. Milliken

Ralph E. Purdy, of, Irvine, California, a citizen of the U.S.A. Post office address: 18 Owen Court, Irvine, CA, 92612-4042 CA

Signature: XXXXXXXXX Date:

Ralph E. Purdy

Attorney Docket No. UCIVN-014US

DECLARATION OF INVENTORSHIP and LIMITED POWER OF ATTORNEY

As a below named inventor, I believe that I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the invention entitled "Continuous Cardiac Perfusion Preservation with PEG-HB for Improved Ischemic Hypothermic Storage" which is described and claimed in Application No. 10/018,534 filed December 17, 2001 for which a patent is sought. My residence, post office address and citizenship are as stated below next to my name.

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I acknowledge my duty under Title 37, Code of Federal Regulations § 1.56(a) to disclose information which is material to the patentability of the invention I am claiming.

☐ I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application: [Parent serial no.], filed [Parent filing date], now [Parent status].

As a named inventor and until I assign my rights to the invention, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith on my behalf: **Frank J. Uxa**, Reg. No. 25,612; **Donald E. Stout**, Reg. No. 34,493; **Robert D. Buyan**, Reg. No. 32,460; **Kenton R. Mullins**, Reg. No. 36,331; **Jo Anne M. Ybaben,** Reg. No. 42,243; **Linda Allyson Fox**, Reg. No.38,883; **Kyle D. Yesland, Ph.D.**, Reg. No. 45,526;**Greg S. Hollrigel, Ph.D.**, Registered Patent Agent, Reg. No. 45,374; **Louise S. Heim**, Registered Patent Agent, Reg. No. 32,337, all of the firm STOUT, UXA, BUYAN & MULLINS, LLP,. **Send correspondence and direct telephone calls to: Robert D. Buyan, Stout, Uxa, Buyan & Mullins, LLP, 4 Venture, Suite 300, Irvine, CA 92618; telephone (949) 450-1750, facsimile (949) 450-1764, email: rbuyan@patlawyers.com**.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that there statements

were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application and any patent issuing thereon.

Danny L. Serna, of, Anaheim, California, a citizen of the U.S.A. Post office address: 7822 East Menton Avenue, Anaheim, CA 92808

Signature: XXXXXXXXXXX Date:
Danny L. Serna

Jeffrey C. Milliken, of, Newport Coast, California, a citizen of the U.S.A. Post office address: 14 Fairwind, Newport Coast, CA, 92687

Signature: _____ Date: 10/25/2002
Jeffrey C. Milliken

Ralph E. Purdy, of, Irvine, California, a citizen of the U.S.A. Post office address: 18 Owen Court, Irvine, CA, 92612-4042

Signature: Ralph E. Purdy Date: 10-29-02
Ralph E. Purdy

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PATENT

Attorney Docket No.: UCIVN-014US

Applicant: Danny L. Serna, Jeffrey C. Milliken, and Ralph E. Purdy
Serial No.: 10/018,534
Filed: 12/17/01
For: Continuous Cardiac Perfusion Preservation with PEG-HB for Improved Ischemic Hypothermic Storage
Group: N/A
Examiner: N/A

POWER OF ATTORNEY BY ASSIGNEE AND EXCLUSION OF INVENTOR(S) UNDER 37 C.F.R. 3.71

Dear Sir:

The undersigned assignee of the entire interest Danny L. Serna, Jeffrey C. Milliken, and Ralph E. Purdy in the above-identified subject application hereby appoints Robert D. Buyan, Reg. No. 32,460 as its attorney to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith, said appointment to be to the exclusion of the inventors and their attorney(s) in accordance with the provisions of 37 C.F.R. 3.71.

An assignment of the entire interest interest Danny L. Serna, Jeffrey C. Milliken, and Ralph E. Purdy in the above-identified subject application:

[ ] was recorded on _____ at reel/frame _____/_____.

[X] is submitted herewith for recording.

Please direct all telephone calls to Robert D. Buyan at 949-450-1750 and all correspondence relative to said application to the following address:

Stout, Uxa, Buyan & Mullins, LLP
4 Venture, Suite 300
Irvine, CA 92618

Dated: October 28, 2002

ASSIGNEE: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

Signature: Linda S. Stevenson

Typed Name: Linda S. Stevenson

Title: Manager, Patent Prosecution

Office of Technology Transfer
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Oakland, CA 94607-5200

UC Case No. 99-375-3

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